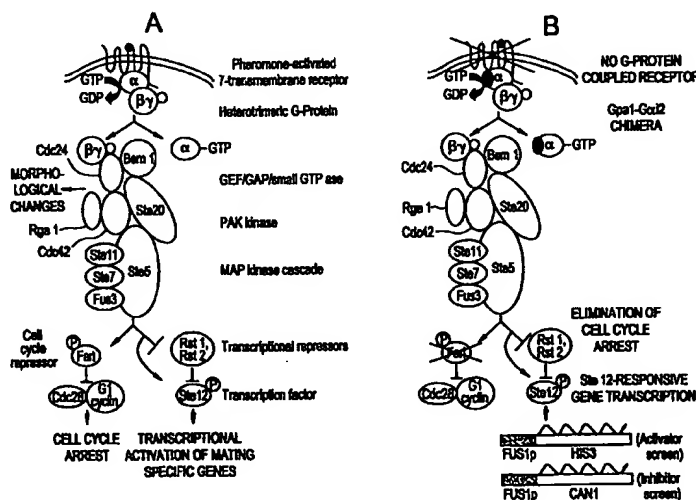




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(54) Title: AGS PROTEINS AND NUCLEIC ACID MOLECULES AND USES THEREFOR



(57) Abstract

A screening assay in yeast is disclosed wherein G-protein coupled-receptor independent activators and inhibitors of the pheromone pathway can be identified using a mammalian cDNA library. Novel Activator of G protein Signaling ("AGS") proteins, which are Ras-related proteins that stimulate G protein activity in a receptor-independent manner, are disclosed, as well as nucleic acid molecules encoding AGS proteins. In addition to isolated AGS proteins, the invention further provides isolated AGS fusion proteins, antigenic peptides and anti-AGS antibodies. The invention also provides isolated AGS nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals in which an AGS gene has been introduced or disrupted. Diagnostic, screening and therapeutic methods utilizing compositions of the invention are also provided.

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AGS PROTEINS AND NUCLEIC ACID MOLECULES AND USES THEREFOR**Background of the Invention**

Heterotrimeric G protein-mediated signal transduction is a tightly regulated event. All known G protein-coupled receptor (GPCR) mediated signaling pathways rely on multiple regulatory mechanisms in order to prevent inappropriate induction of the signal and to facilitate recovery during chronic stimulation (Gilman (1987) *Ann. Rev. Biochem.* 56:615-649, reviewed in Simon et al. (1991) *Science* 252:802-808; Conklin and Bourne (1993) *Cell* 73:631-641; Neer (1995) *Cell* 80:249-257; Rens-Domiano and Hamm (1995) *FASEB J.* 9:1059-1066). These regulatory mechanisms function at every level of the signaling cascade. Regulation of GPCR activation is believed to involve phosphorylation of the receptor C-terminus and subsequent receptor internalization (Palczewski and Benkovic (1991) *Trends Biol. Sci.* 16:387-391; Goodman et al. (1996) *Nature* 383:447-450; Chen and Konopka (1996) *Mol. Cell. Biol.* 16:247-257), though this does not appear to be a universal mechanism (Daunt et al. (1997) *Mol. Pharm.* 51:711-720). Known mechanisms of regulation of signal transduction at the level of the heterotrimeric G protein include receptor-mediated facilitation of GTP/GDP exchange on G α (reviewed in Simon et al. (1991) *Science* 252:802-808; Conklin and Bourne (1993) *Cell* 73:631-641; Neer (1995) *Cell* 80:249-257; Rens-Domiano and Hamm (1995) *FASEB J.* 9:1059-1066) and enhancement of the intrinsic GTPase activity of G α proteins by RGS-like proteins (reviewed in Berman and Gilman (1998) *J. Biol. Chem.* 273:1269-1272). Activation of PAKs, serine/threonine kinases that transduce signals from heterotrimeric G proteins to the MAP kinase cascade, has been shown to occur through interaction with either the small G proteins Cdc42 and Rac, or through interaction with heterotrimeric G proteins (reviewed in Sells and Chernoff (1997) *Trends Cell Biol.* 7:162-167). GPCR-coupled MAP kinase cascades and their downstream transcription factors, in turn, are regulated through phosphorylation/dephosphorylation cycles that may or may not require small G proteins (reviewed in Cobb and Goldsmith (1995) *J. Biol. Chem.* 270:14843-14846). Non-receptor activators of G-proteins have also been identified. These include both protein activators (Strittmatter et al. (1993) *Proc. Nat'l Acad. Sci., USA* 90:5327-5331; Okamoto et al. (1995) *J. Biol. Chem.*

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270:4205-4208; Sato et al. (1996) *J. Biol. Chem.* 271:30052-30060) and non-protein activators (summarized in Odagaki et al. (1998) *Life Sciences* 62:1537-1541.

Even with the identification of these diverse regulatory systems, an in-depth understanding of the temporal and spatial regulation of GPCR mediated signaling remains elusive. In fact, cellular variations in the efficiency and/or specificity of coupling observed for many specific receptor-heterotrimeric G protein complexes suggest the presence of additional unidentified, cell-specific regulators of the signaling process.

10 Summary of the Invention

In an attempt to identify novel factors involved in regulating signaling through GPCR-mediated signal transduction pathways, a screening system was devised in the yeast *Saccharomyces cerevisiae* designed to identify receptor-independent activators of the pheromone response pathway. These functional screens can be designed to target not only specific regulatory pathways in yeast, but also an introduced mammalian component or components. Yeast strains containing an intact signal transduction cascade but lacking a functional GPCR were made conditional for growth upon either pheromone pathway activation (activator screen) or pheromone pathway inactivation (inhibitor screen). In addition, the activator yeast strain carries an integrated *FUS1p-HIS3* construct, making histidine prototrophy conditional upon pheromone pathway activation. The inhibitor yeast strain carries an episomal *FUS1p-CAN1* construct. Adult human liver cDNAs expressed in a yeast vector under control of a galactose-inducible promoter were introduced into these strain, and those cDNAs that conferred growth in a galactose- and insert-dependent fashion were identified. Provided herein is the characterization of one member of these activator cDNAs, encoding a protein of 281 amino acids with significant homology to ras-related G proteins and containing alterations in conserved amino acids consistent with a deficiency in GTP hydrolysis activity (i.e., a constitutively active form of ras-related G protein). Genetic epistasis tests in yeast were consistent with this activator functioning at the level of the heterotrimeric G-protein and facilitating GTP exchange on the chimeric G α i2. This protein is referred to herein as Activator of G protein Signaling, or "AGS", or

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AGS1AGS1 also shows G α selectivity, as measured by growth assays in yeast expressing various mammalian G α constructs, and tissue specific expression, as measured by Northern blot analysis. A cDNA product identified from the inhibitor screen encodes a previously identified regulator of G-protein signaling, human RGS5.

5 In one embodiment, an isolated nucleic acid molecule of the present invention encodes an AGS protein (*e.g.*, the nucleic acid molecule has a nucleotide sequence having at least 86% identity to the nucleotide sequence of SEQ ID NO: 1 or the complement thereof). In another embodiment, an isolated nucleic acid molecule of the present invention has a nucleotide sequence having at least 90% identity to the
10 nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 3, or the complement of SEQ ID NO: 1 or SEQ ID NO: 3. In yet another embodiment, the isolated nucleic acid molecule has the nucleotide sequence of SEQ ID NO: 1, or the complement thereof. In another embodiment, the isolated nucleic acid molecule has the nucleotide sequence of SEQ ID NO: 3, or the complement thereof. In yet another embodiment, an isolated nucleic acid
15 molecule of the present invention encodes a protein that activates G protein-coupled signal transduction in a G protein-coupled receptor independent manner.

In another embodiment, an isolated nucleic acid molecule of the present invention has a nucleotide sequence which encodes a protein having an amino acid sequence at least 97% identical to the amino acid sequence of SEQ ID NO: 2. In another
20 embodiment, the isolated nucleic acid molecule encodes a protein having the amino acid sequence of SEQ ID NO: 2. In yet another embodiment, an isolated nucleic acid molecule of the present invention encodes a protein which activates G protein-coupled signal transduction in a G protein-coupled receptor independent manner.

The present invention also provides vectors including nucleic acid sequences
25 which encode all or a portion of an AGS protein as well as host cells including such vectors. The invention further provides methods for producing an AGS protein including culturing host cells which express an AGS protein. The invention also provides transgenic animals which contain cells carrying a transgene encoding AGS protein.

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In another embodiment, the present invention provides isolated AGS proteins (*e.g.*, an isolated AGS protein having an amino acid sequence at least 97% identity to the amino acid sequence of SEQ ID NO: 2.) In another embodiment, the protein has the amino acid sequence of SEQ ID NO: 2. The present invention also provides fusion
5 proteins having at least a portion of an AGS protein operatively linked to a non-AGS polypeptide as well as antibodies that specifically bind to an AGS protein (*e.g.*, monoclonal or polyclonal antibodies). The invention further provides pharmaceutical compositions including AGS proteins or AGS-antibodies.

The present invention further provides methods for identifying compounds that
10 modulate cellular signal transduction. In one embodiment, the method includes the steps of (a) contacting a cell that expresses an AGS protein with a test compound; (b) determining the effect of the test compound on the activity of the AGS protein; and (c) identifying the test compound as a modulator of signal transduction based on the ability of the compound to modulate the activity of the AGS protein in the cell. In another
15 embodiment, the AGS proteins utilized in the subject methods have an amino acid sequence which is at least 97% identical to SEQ ID NO: 2 and stimulates G protein activity in a receptor-independent manner. In yet another embodiment, the AGS protein used in the subject methods has the amino acid sequence of SEQ ID NO: 2.

In yet another embodiment, the compound identified by the above method is a
20 nucleic acid encoding a polypeptide capable of inhibiting the activity of the AGS protein. In still another embodiment, the above method further comprises a nucleic acid encoding an inhibitor of the AGS protein. In a preferred embodiment, the above method is suitable for identifying a test compound capable of modulating the activity of the AGS protein by modulating the inhibitor of the AGS protein.

25 In a preferred embodiment, cells used in the screening methods of the present invention have been engineered to express the AGS protein. Preferred cells for use in the screening methods are yeast cells. In another preferred embodiment, the yeast cells have further been engineered to express a G protein α subunit, a chimeric G protein α subunit, or a G α 1-G α i2 chimeric G protein α subunit. The activity of a test compound
30 on a cell-associated activity (*e.g.*, a G-protein mediated activity) can be determined by monitoring a pheromone response pathway in the yeast cells (*e.g.*, by measuring the

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activity of a pheromone responsive promoter in the yeast cells), by monitoring the ability of the test compound to bind to the AGS protein, or by monitoring the ability of the test compound to modulate the interaction of the AGS protein with a target molecule (e.g., a G protein).

5 The present invention further provides methods for modulating G protein-coupled signal transduction in a cell (e.g., by contacting a cell with an agent which modulates AGS protein activity or AGS nucleic acid expression). Methods for treating a subject having a disorder characterized by aberrant AGS protein activity or nucleic acid expression are also provided as well as methods for detecting the presence of AGS in a
10 biological sample.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

15 *Figures 1A and 1B* depict diagrams of the yeast pheromone response pathway with major signaling components, (Abbr. indicated are: α , Gpa1; β , Ste4; γ , Ste18; P, phosphorylation) and the modifications made to the pheromone response pathway (Fig. 1B) for the Activator screen (i.e., strains CY1316/1183) and the Inhibitor screen (e.g., strains CY1141/1451-AGS1/2440).

20 *Figure 2* depicts the major steps in carrying out the yeast Activator (left panel) and Inhibitor (right panel) screens.

Figures 3A and 3B depict the coding region of the cDNA sequence (Fig. 3A) and predicted amino acid sequence of human AGS (Fig. 3B). The nucleotide sequence corresponds to SEQ ID NO: 1. The amino acid sequence corresponds to SEQ ID NO:2.
25 The composite insert region of cDNA clone pYES2-L1 is available from GenBank, Accession No. #AF069506. Consensus sequences for ras-like G proteins (Valencia, et al. (1991) *Biochemistry* 30:4637) are underlined. Regions unique to AGS1 are indicated in *italics*. Three residues that are normally conserved in ras-like G proteins but divergent in AGS1 and Rnd1-3 (Nobes, et al. (1998) *J. Cell Biol.* 141:187) are indicated
30 with asterisks below the residue. These divergent residues were shown to confer GTPase deficiency in Rnd1.

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Figures 4A and 4B depict the alignment of AGS with representatives of all major classes of small G proteins in humans indicating that AGS is likely to be the founding member of a novel class of small G proteins in humans.

Figure 5 depicts the alignment of residues in the P region and in the G' region of various small G proteins. Asterisks indicate the location of three highly conserved residues that are altered in AGS, Rnd1, Rnd2, and Rnd3. Numbers indicate the positions of the amino acid sequence of AGS.

Detailed Description of the Invention

The present invention is based on the discovery of nucleic acid and protein molecules, referred to herein as Activator of G protein Signaling ("AGS") proteins and nucleic acid molecules, which play a role in or function in G protein-mediated signal transduction in the absence of receptor stimulation. These molecules were identified using an assay of the invention that employs yeast-based functional screens using the pheromone response pathway. In part, the assay relies on the observation that a G-protein coupled receptor (GPCR) signaling pathway is required for mating in haploid yeast. Moreover, there is functional redundancy between this pathway and the mammalian GPCR signaling pathway and all of the major signaling components and regulatory systems in GPCR-mediated signal transduction in mammalian systems appears to be conserved in the yeast pheromone response pathway (Figure 1A) (Kurjan (1993) *Annu. Rev. Genet.* 27:147-179; Bardwell et al., *Dev. Biol.* 166:363-379). Thus, this assay can be used to search for mammalian regulators of this system. Normally, upon receptor activation by pheromone, the GPCR associated heterotrimeric G-protein undergoes subunit dissociation into GTP-bound activated $G\alpha$ (Gpa1) and $G\beta\gamma$ (Ste4/Ste18). Free $G\beta\gamma$ dimer then transduces a signal through a p21-activated kinase (Ste20) to a MAP kinase cascade, leading to the transcriptional activation of mating-specific genes by the transcription factor Ste12, as well as Far1-mediated growth arrest in the G_1 phase of the cell cycle. For the screening assay of the present inventions, the pheromone response pathway was engineered to create yeast strains that could be made conditional for growth upon either pheromone pathway activation or suppression (Figure 1B). Using these strains, functional screens were developed to identify mammalian

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cDNAs whose expression either activates or down-regulates the yeast pheromone response pathway independent of the presence of receptor. A human AGS cDNA was isolated in a functional cloning screen in yeast based upon its ability to activate G protein signaling in a manner independent of G protein-coupled receptor stimulation.

5 Genetic evidence (described in detail in Examples 1-3) indicates that this AGS-dependent activation occurs at the level of the heterotrimeric G protein. Thus, in one embodiment, the AGS molecules stimulate the activity of one or more G proteins involved in a G protein-mediated signal transduction pathway, *e.g.*, a pheromone response cascade in yeast, to thereby activate G protein-mediated signal transduction

10 independent of G protein coupled receptor stimulation. In a preferred embodiment, the AGS molecules of the present invention stimulate the activity of one or more G proteins involved in a G protein-mediated signal transduction pathway, such that G protein coupled receptor-mediated signal transduction is amplified. In a particularly preferred embodiment, the AGS molecules are capable of modulating the activity of G α subunits,

15 such as a mammalian G α i2 subunit or a chimeric G α subunit comprising a portion of the yeast Gpa1 protein (*e.g.*, the amino-terminal 41 amino acids) linked to a mammalian G α i2 subunit.

Since the AGS proteins of the invention can function in activation of the pheromone response cascade in yeast cells, and potentially modulate the MEK pathway

20 in mammalian cells, the AGS molecules of the present invention can be used in methods for identifying antagonists of G protein signaling, either receptor-dependent or receptor independent, in screening assays in host cells, such as mammalian or yeast host cells.

A particularly preferred AGS nucleic acid and protein, depicted in Figure 5 (and shown in SEQ ID NO: 1 and 2, respectively), is isolated from human cells. Figure 5

25 depicts the nucleotide sequence of the coding region of an AGS cDNA which was isolated from a human liver cDNA library. An AGS cDNA nucleotide sequence that includes 5' and 3' untranslated regions is shown in SEQ ID NO: 3. The cDNA sequence encodes a predicted protein which is 281 amino acid residues in length and which exhibits homology to ras-related G proteins. The AGS protein also contains alterations

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in amino acids that typically are conserved among ras-related G proteins that are consistent with AGS having a deficiency in GTP hydrolysis activity.

The AGS proteins of the invention contain several motifs characteristic of Ras superfamily members, including the phosphate/magnesium binding regions
5 GXXXXGK(S/T)(SEQ ID NO: 18) (the P-loop) and DXXG (SEQ ID NO: 19) (the G' region), as well as the guanine base binding loops NKXD (SEQ ID NO: 20) and EXSAK (SEQ ID NO: 21) (Bourne et al. (1991) *Nature* 349:117-127; Valencia et al. (1991) *Biochemistry* 30:4637-4648). Additionally, the motif regions G-1 through G-5, characteristic of GTPases (Bourne et al. (1991) *Nature* 349:117-127), are present in
10 AGS. Moreover, the C-terminal region of AGS has a typical CAAX (SEQ ID NO: 22) motif (Bourne et al. (1991) *Nature* 349:117-127; Valencia et al. (1991) *Biochemistry* 30:4637-4648; Del Villar et al. (1996) *Biochem. Soc. Trans.* 24:709-713). The CAAX motif is immediately preceded by a basic region QAKDKER (SEQ ID NO: 23), thought to be important in anchoring ras-like G proteins to the phospholipid bilayer (Magee and
15 Newman (1992) *Trends Cell Biol.* 2:318-323).

Various aspects of the invention are described in further detail in the following subsections:

20 I. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode an AGS protein or biologically active portion thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify AGS-encoding nucleic acid (*e.g.*, AGS mRNA). As used herein, the term "nucleic acid molecule" is intended to
25 include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic
30 acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*e.g.*, sequences located at the 5' and 3' ends of the nucleic acid) in the

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genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated AGS nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO: 3, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, a human AGS cDNA can be isolated from a human liver cDNA library using all or portion of SEQ ID NO:1 or SEQ ID NO: 3 as a hybridization probe and standard hybridization techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1 or SEQ ID NO: 3 can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO:1 or SEQ ID NO:3. For example, mRNA can be isolated from liver cells (*e.g.*, by the guanidinium-thiocyanate extraction procedure of Chirgwin et al. (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (*e.g.*, Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding

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to an AGS nucleotide sequence can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1. The sequence of SEQ ID NO:1 corresponds to the coding region of an AGS cDNA isolated from human liver cells. Another preferred AGS cDNA sequence is shown in SEQ ID NO: 3, which includes 5' and 3' untranslated regions. This cDNA comprises sequences encoding the AGS protein (*e.g.*, "the coding region", from nucleotides 154 to 999), as well as 5' untranslated sequences (nucleotides 7 to 153) and 3' untranslated sequences (nucleotides 1000 to 1801).

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is the complement of the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3 is one which has a nucleotide sequence that directly pairs with that of SEQ ID NO: 1 or 3, according to the rules of Watson and Crick base pairing, wherein A pairs with T and G pairs with C. For example, the complement of the sequence 5' GGATGC 3' would be 3'CCTACG 5" (which, written in the standard 5' to 3' direction, would be 5' GCATCC 3').

In still another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which is at least 60%, preferably at least 70%, more preferably at 80%, and even more preferably at least 90%, or 95%, or 96%, or 97%, or 98%, or 99% homologous to the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3, or a portion of any of these nucleotide sequences. In an additional preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions, to the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3, or a portion of any of these nucleotide sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the coding region of SEQ ID NO:1 or SEQ ID NO:3, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of

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an AGS protein. The nucleotide sequence determined from the cloning of the AGS genes from a mammal, *e.g.*, humans, allows for the generation of probes and primers designed for use in identifying and/or cloning AGS homologues in other cell types, *e.g.* from other tissues, as well as AGS homologues from other mammals, *e.g.*, mice, rats, etc. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of SEQ ID NO:1 or SEQ ID NO:3 sense, an anti-sense sequence of SEQ ID NO:1 or SEQ ID NO:3, or naturally occurring mutants thereof. Primers based on the nucleotide sequence in SEQ ID NO:1 or SEQ ID NO:3 can be used in PCR reactions to clone AGS homologues. Probes based on the AGS nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.* the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress an AGS protein, such as by measuring a level of an AGS-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting AGS mRNA levels or determining whether a genomic AGS gene has been mutated or deleted.

In one embodiment, the nucleic acid molecule of the invention encodes a protein or portion thereof which includes an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains the ability to modulate a G-protein mediated response in a cell. As used herein, the language "sufficiently homologous" refers to proteins or portions thereof which have amino acid sequences which include a minimum number of identical or equivalent (*e.g.*, an amino acid residue which has a similar side chain as an amino acid residue in SEQ ID NO:2) amino acid residues to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof is able to modulate a G-protein mediated response in a cell. In another embodiment, the protein is at least 60%, preferably at least 70%, more preferably at least 80%, more preferably at least 90% and most preferably at

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least 95%, or 96%, or 97%, or 98%, or 99% homologous to the entire amino acid sequence of SEQ ID NO:2.

Portions of proteins encoded by the AGS nucleic acid molecule of the invention are preferably biologically active portions of the AGS protein. As used herein, the term

5 "biologically active portion of AGS" is intended to include a portion, *e.g.*, a domain/motif, of AGS that has one or more of the following activities: 1) it can interact with (*e.g.*, bind to) a G protein; 2) it can modulate the activity of a G protein; 3) it can interact with (*e.g.*, bind to) a G protein target molecule; 4) it can modulate the activity of a G protein target molecule; 5) it can modulate a G protein-mediated response in a cell,

10 independent of G protein-coupled receptor activation; and 6) it can augment G protein-coupled receptor signaling by modulating a G protein-mediated response in a cell. Standard binding assays, *e.g.*, immunoprecipitations, yeast two-hybrid assays, and *in vitro* column binding assays, as described herein, can be performed to determine the ability of an AGS protein or a biologically active portion thereof to interact with (*e.g.*,

15 bind to) a G protein. To determine whether an AGS protein or biologically active portion thereof can modulate G-protein mediated response in a cell, the AGS protein or biologically active portion thereof can be introduced into a cell (*e.g.*, transformed or transfected) which has been engineered to grow only in the presence of an AGS protein or biologically active portion thereof, *e.g.*, yeast cell strain 1316/1183 (described in the

20 Examples) and the ability of the AGS protein or biologically active portion thereof to facilitate growth determined. Alternatively, a cell can be transformed or transfected with a G-protein mediated signal transduction responsive reporter construct (*e.g.*, FUS1-luciferase) which responds to G-protein mediated signaling by expressing luciferase, and a nucleic acid encoding the AGS protein or biologically active portion thereof. The cells

25 can be harvested and lysed and reporter activity, *e.g.*, luciferase activity, can be measured and compared to reporter activity in a control cell. Examples of control cells include cells which include the G-protein mediated signal transduction responsive reporter construct. An alteration in reporter activity in the cells which include nucleic acid encoding the AGS protein, as compared to reporter activity in the cells without

30 nucleic acid encoding the AGS protein is indicative of a modulation of a G-protein mediated response in the cell.

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In addition to the AGS nucleotide sequences shown in SEQ ID NO:1 and SEQ ID NO:3, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of AGS may exist within a population. Such genetic polymorphism in the AGS gene may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding an AGS protein, preferably a mammalian AGS protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the AGS gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in AGS that are the result of natural allelic variation and that do not alter the functional activity of AGS are intended to be within the scope of the invention. Moreover, nucleic acid molecules encoding AGS proteins from other species, and thus which have a nucleotide sequence which differs from the sequence of SEQ ID NO:1 or SEQ ID NO:3, are intended to be within the scope of the invention. For example, non-human homologues of the AGS cDNAs of the invention can be isolated based on their homology to the AGS nucleic acid disclosed herein using the human cDNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 15 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3. In other embodiments, the nucleic acid is at least 30, 50, 100, 250 or 500 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 65%, more preferably at least about 70%, and even more preferably at least about 75% or more homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C,

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followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 or SEQ ID NO:3 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule
5 refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein). In one embodiment, the nucleic acid encodes a natural human AGS.

In addition to naturally-occurring allelic variants of the AGS sequences that may exist in the population, the skilled artisan will further appreciate that changes can be
10 introduced by mutation into the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3, thereby leading to changes in the amino acid sequence of the encoded AGS proteins, without altering the functional activity of the AGS proteins. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of SEQ ID NO:1 or SEQ ID NO:3. A "non-essential"
15 amino acid residue is a residue that can be altered from the wild-type sequences of AGS (*e.g.*, the sequence of SEQ ID NO:2) without altering the activity of AGS, whereas an "essential" amino acid residue is required for AGS activity. For example, conserved amino acid residues in the following motifs that are conserved among Ras family members are most likely important for the activity of an AGS protein and are thus
20 essential residues of AGS: the phosphate/magnesium binding regions GXXXXGK(S/T)(SEQ ID NO: 18) (the P-loop) and DXXG (SEQ ID NO: 19), the guanine base binding loops NKXD (SEQ ID NO: 20) and EXSAK (SEQ ID NO: 21), the motif regions G-1 through G-5, characteristic of GTPases, and the C-terminal CAAX (SEQ ID NO: 22) motif. Other amino acid residues, however, (*e.g.*, those that are not
25 conserved or only semi-conserved family of ras-related small G proteins) may not be essential for activity and thus are likely to be amenable to alteration without altering AGS activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding AGS proteins that contain changes in amino acid residues that are not essential
30 for AGS activity. Such AGS proteins differ in amino acid sequence from SEQ ID NO:2, yet retain at least one of the AGS activities described herein. In one embodiment, the

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isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2, and is capable of modulating a G-protein mediated response in a cell. Preferably, the protein encoded by the nucleic acid molecule is at least about 70% homologous to SEQ ID NO:2, more preferably at least about 80-85% homologous, even more preferably at least about 90-95% homologous, and most preferably at least about 95, 96, 97, 98, or 99% homologous to SEQ ID NO:2.

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first or second sequence for optimal alignment). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (*e.g.*, as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (*e.g.*, % homology = # of identical positions/total # of positions x 100). This number can be modified if using an alignment algorithm which allows for gaps. Alternatively, a percent similarity between two sequences can be determined wherein a non-identical pair of, for example, amino acids sharing a position are evolutionary conserved. Such a comparison can be made utilizing an algorithm which relies on a residue weight table, for example, a PAM residue weight table (*e.g.*, PAM 120 or PAM 150) or a BLOSUM weight residue table (*e.g.* BLOSUM 62).

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68,

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modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain

5 nucleotide sequences homologous to AGS nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to ANTIKINE protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids*

10 *Research* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN

15 program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Another preferred, non-limiting example of a mathematical algorithm utilized for the alignment of protein sequences is the Lipman-Pearson algorithm (Lipman and Pearson

20 (1985) *Science* 227:1435-1441). When using the Lipman-Pearson algorithm, a PAM250 weight residue table, a gap length penalty of 12, a gap penalty of 4, and a Ktuple of 2 can be used. A preferred, non-limiting example of a mathematical algorithm utilized for the alignment of nucleic acid sequences is the Wilbur-Lipman algorithm (Wilbur and Lipman (1983) *Proc. Natl. Acad. Sci. USA* 80:726-730). When using the Wilbur-

25 Lipman algorithm, a window of 20, gap penalty of 3, Ktuple of 3 can be used. Both the Lipman-Pearson algorithm and the Wilbur-Lipman algorithm are incorporated, for example, into the MEGALIGN program (*e.g.*, version 3.1.7) which is part of the DNASTAR sequence analysis software package. Alternatively, a PAM 250 residue weight Table, a GAP penalty of 10, and a GAP length penalty of 10 can be used.

30 Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Wilbur-Lipmann which is part of

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MegAlign* sequence alignment software. When utilizing the Wilbur-Lipmann algorithm, a K-tuple of 1, a GAP penalty of 3, a window of 5, and diagonals saved set to = 5 can be used. Multiple alignment can be performed using the Clustal algorithm.

An isolated nucleic acid molecule encoding an AGS protein homologous to the protein of SEQ ID NO:2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1 or SEQ ID NO:3, by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in an AGS protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of an AGS coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for an AGS activity described herein to identify mutants that retain AGS activity. Following mutagenesis of SEQ ID NO:1 or SEQ ID NO:3, the encoded protein can be expressed recombinantly and the activity of the protein can be determined using, for example, assays described herein.

In addition to the nucleic acid molecules encoding AGS proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*,

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complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire AGS coding strand, or to only a portion thereof. In one
5 embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding an AGS protein. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide
10 sequence encoding AGS. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*e.g.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding AGS proteins disclosed herein (*e.g.*, SEQ ID NO:1 and SEQ ID NO: 3), antisense nucleic acids of the invention can be
15 designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of an AGS mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of an AGS mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of an AGS mRNA.
20 An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or
25 variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used.

Alternatively, the antisense nucleic acid can be produced biologically using an
30 expression vector into which a nucleic acid has been subcloned in an antisense orientation (*e.g.*, RNA transcribed from the inserted nucleic acid will be of an antisense

orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA
5 and/or genomic DNA encoding an AGS protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. An example of a route of administration of an antisense nucleic acid molecule of the invention includes direct injection at a tissue site. Alternatively, an antisense nucleic acid molecule can be modified to target selected cells and then administered systemically. *e.g.* The antisense
10 nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention
15 is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-*o*-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a
20 chimeric RNA-DNA analogue (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in
25 Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave AGS mRNA transcripts to thereby inhibit translation of AGS mRNAs. A ribozyme having specificity for an AGS-encoding nucleic acid can be designed based upon the nucleotide sequence of an AGS cDNA disclosed herein. (see, *e.g.*, Cech et al. U.S. Patent No. 4,987,071 and Cech et al. U.S. Patent No. 5,116,742). Alternatively, AGS
30 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity

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from a pool of RNA molecules. (see, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418).

Alternatively, AGS gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of an AGS gene (*e.g.*, an AGS promoter and/or enhancer) to form triple helical structures that prevent transcription of an AGS gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. et al. (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

10 **II. Recombinant Expression Vectors and Host Cells**

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding an AGS protein (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses) as well as baculoviral vectors, which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is

5 operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory

10 sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).*e.g.* It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of

15 the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, AGS proteins, mutant forms of AGS proteins, fusion proteins, etc.).

20 Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. In one embodiment, the coding sequence of an AGS protein is cloned into a

25 pGEX expression vector to create a vector encoding a fusion protein comprising, from the N-terminus to the C-terminus, GST-thrombin cleavage site-AGS protein. The fusion protein can be purified by affinity chromatography using glutathione-agarose resin. Recombinant AGS protein unfused to GST can be recovered by cleavage of the fusion protein with thrombin.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, an AGS expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, an AGS protein can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165), pBlueBacHis2 (Invitrogen Corporation, San Diego, CA), and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pcDNA3.1/His (Invitrogen Corporation, San Diego, CA), pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning:*

A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to an AGS mRNA. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. A host cell can be any prokaryotic or eukaryotic cell. For example, AGS protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or

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mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989*), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding AGS or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*e.g.*, express) an AGS protein. Accordingly, the invention further provides methods for producing AGS proteins using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding an AGS protein has been introduced) in a suitable medium until AGS protein is produced. In another embodiment, the method further comprises isolating AGS protein from the medium or the host cell.

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The host cells of the invention can also be used to produce nonhuman transgenic animals. The nonhuman transgenic animals can be used in screening assays designed to identify agents or compounds, *e.g.*, drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental symptoms of selected disorders such as cardiovascular disorders and proliferative disorders. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which AGS-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous AGS sequences have been introduced into their genome or homologous recombinant animals in which endogenous AGS sequences have been altered. Such animals are useful for studying the function and/or activity of AGS and for identifying and/or evaluating modulators of AGS activity. As used herein, a "transgenic animal" is a nonhuman animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include nonhuman primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a nonhuman animal, preferably a mammal, more preferably a mouse, in which an endogenous AGS gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing AGS-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The AGS cDNA sequence of SEQ ID NO:1 can be used as a transgene. Alternatively, a non-human homologue of the AGS genes can be isolated based on hybridization to the AGS cDNA (*e.g.*, hybridization to SEQ ID NO:1, described further in subsection I above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency

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of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to an AGS transgene to direct expression of AGS protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are

5 described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Patent No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of an AGS

10 transgene in its genome and/or expression of AGS mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding AGS can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains

15 at least a portion of an AGS gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the AGS gene. The AGS gene can be a human gene (*e.g.*, SEQ ID NO:1), but more preferably, is a nonhuman homologue of a human AGS gene (*e.g.*, a murine homologue). For example, a mouse AGS gene can be used to construct a homologous recombination vector suitable for altering an

20 endogenous AGS gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous AGS gene is functionally disrupted (*e.g.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous AGS gene is mutated or otherwise altered

25 but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous AGS protein). In the homologous recombination vector, the altered portion of the AGS gene is flanked at its 5' and 3' ends by additional nucleic acid of the AGS gene to allow for homologous recombination to occur between the exogenous AGS gene carried by the vector and an endogenous AGS

30 gene in an embryonic stem cell. The additional flanking AGS nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene.

Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced

5 AGS gene has homologously recombined with the endogenous AGS gene are selected (see *e.g.*, Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be

10 implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described

15 further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec et al.; WO 91/01140 by Smithies et al.; WO 92/0968 by Zijlstra et al.; and WO 93/04169 by Berns et al.

In another embodiment, transgenic nonhumans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One

20 example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso et al. (1992) *PNAS* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals

25 containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Clones of the nonhuman transgenic animals described herein can also be produced according to the

30 methods described in Wilmut, I. et al. (1997) *Nature* 385:810-813.

III. Isolated AGS Proteins and Anti- AGS Antibodies

Another aspect of the invention pertains to isolated AGS proteins, and biologically active portions thereof, as well as peptide fragments suitable for use as immunogens to raise anti- AGS antibodies. An "isolated" or "purified" protein or

5 biologically active portion thereof is substantially free of cellular material when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of AGS protein in which the protein is separated from cellular components of the cells in which it is naturally or recombinantly produced. In one

10 embodiment, the language "substantially free of cellular material" includes preparations of AGS protein having less than about 30% (by dry weight) of non- AGS protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non- AGS protein, still more preferably less than about 10% of non- AGS protein, and most preferably less than about 5% non- AGS protein. When the AGS protein or

15 biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *e.g.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The language "substantially free of chemical precursors or other chemicals" includes preparations of AGS protein in which the protein

20 is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of AGS protein having less than about 30% (by dry weight) of chemical precursors or non- AGS chemicals, more preferably less than about 20% chemical precursors or non- AGS chemicals, still

25 more preferably less than about 10% chemical precursors or non- AGS chemicals, and most preferably less than about 5% chemical precursors or non- AGS chemicals. In preferred embodiments, isolated proteins or biologically active portions thereof lack contaminating proteins from the same animal from which the AGS protein is derived. Typically, such proteins are produced by recombinant expression of, for example, a

30 human AGS protein in a nonhuman cell.

An isolated AGS protein or a portion thereof of the invention can modulate a G-protein mediated response in a cell. In preferred embodiments, the protein or portion thereof comprises an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains the ability to modulate a G-protein mediated response in a cell. The portion of the protein is preferably a biologically active portion as described herein. In another preferred embodiment, the AGS protein has an amino acid sequence shown in SEQ ID NO:2. In yet another preferred embodiment, the AGS protein has an amino acid sequence which is encoded by a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions, to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3. In still another preferred embodiment, the AGS protein has an amino acid sequence which is encoded by a nucleotide sequence that is at least 60%, preferably at least 70%, more preferably at least 80%, and even more preferably at least 90%, or 95%, or 96%, or 97%, or 98%, or 99% homologous to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO: 3. The preferred AGS proteins of the present invention also preferably possess at least one of the AGS activities described herein. For example, a preferred AGS protein of the present invention includes an amino acid sequence encoded by a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions, to the nucleotide sequence SEQ ID NO:1 or SEQ ID NO: 3 and which can modulate a G-protein mediated response in a cell.

In other embodiments, the AGS protein is substantially homologous to the amino acid sequence of SEQ ID NO:2 and retains the functional activity of the protein of SEQ ID NO:2 yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the AGS protein is a protein which comprises an amino acid sequence which is at least 60%, preferably at least 80%, and more preferably at least 86%, or 88%, or 90%, and most preferably at least 95%, or 96%, or 97%, or 98%, or 99% homologous to the entire amino acid sequence of SEQ ID NO:2 and which has at least one of the AGS activities described herein. In other embodiments, the invention pertains to a full length human protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2.

- 30 -

Biologically active portions of the AGS protein include peptides comprising amino acid sequences derived from the amino acid sequence of the AGS protein, *e.g.*, the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence of a protein homologous to the AGS protein, which include less amino acids than the full length AGS protein or the full length protein which is homologous to the AGS protein, and exhibit at least one activity of the AGS protein. Typically, biologically active portions (peptides, *e.g.*, peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, 50, 100 or more amino acids in length) comprise a domain or motif, with at least one activity of the AGS protein. In a preferred embodiment, the biologically active portion of the protein includes a motif selected from the following: the phosphate/magnesium binding regions GXXXXGK(S/T)(SEQ ID NO: 18) (the P-loop) and DXXG (SEQ ID NO: 19), the guanine base binding loops NKXD (SEQ ID NO: 20) and EXSAK (SEQ ID NO: 21) the motif regions G-1 through G-5, characteristic of GTPases, the C-terminal CAAX (SEQ ID NO: 22) motif, and/or the QAKDKER motif (SEQ ID NO:23) and can modulate the activity of a G-protein. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the activities described herein. Preferably, the biologically active portions of the AGS protein include one or more selected domains/motifs or portions thereof having biological activity.

AGS proteins are preferably produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the AGS protein is expressed in the host cell. The AGS protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, an AGS protein, polypeptide, or peptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native AGS protein can be isolated from cells, for example using an anti- AGS antibody (described further below).

The invention also provides AGS chimeric or fusion proteins. As used herein, an AGS "chimeric protein" or "fusion protein" comprises an AGS polypeptide operatively linked to a non- AGS polypeptide. An " AGS polypeptide" refers to a polypeptide

having an amino acid sequence corresponding to AGS, whereas a "non- AGS polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the AGS protein, *e.g.*, a protein which is different from the AGS protein and which is derived from the same or a different
5 organism. Within the fusion protein, the term "operatively linked" is intended to indicate that the AGS polypeptide and the non- AGS polypeptide are fused in-frame to each other. The non- AGS polypeptide can be fused to the N-terminus or C-terminus of the AGS polypeptide. For example, in one embodiment the fusion protein is a GST-
10 AGS fusion protein in which the AGS sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant AGS. In another embodiment, the fusion protein is an AGS protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of AGS can be increased through use of a heterologous signal sequence.

15 Preferably, an AGS chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini,
20 filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene
25 fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). An AGS-encoding nucleic acid can be cloned into such an expression vector such that the fusion
30 moiety is linked in-frame to the AGS protein.

The present invention also pertains to homologues of the AGS proteins which function as either an AGS agonist (mimetic) or an AGS antagonist. In a preferred embodiment, the AGS agonists and antagonists stimulate or inhibit, respectively, a subset of the biological activities of the naturally occurring form of the AGS protein.

5 Thus, specific biological effects can be elicited by treatment with a homologue of limited function. In one embodiment, treatment of a subject with a homologue having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the AGS protein.

10 Homologues of the AGS protein can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of the AGS protein. As used herein, the term "homologue" refers to a variant form of the AGS protein which acts as an agonist or antagonist of the activity of the AGS protein. An agonist of the AGS protein can retain substantially the same, or a subset, of the biological activities of the AGS protein. An antagonist of the
15 AGS protein can inhibit one or more of the activities of the naturally occurring form of the AGS protein, by, for example, competitively binding to a G-protein or downstream or upstream member of the pheromone response cascade.

In an alternative embodiment, homologues of the AGS protein can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of the AGS
20 protein for AGS protein agonist or antagonist activity. In one embodiment, a variegated library of AGS variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of AGS variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential AGS
25 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of AGS sequences therein. There are a variety of methods which can be used to produce libraries of potential AGS homologues from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the
30 synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the

desired set of potential AGS sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477.

5 In addition, libraries of fragments of the AGS protein coding can be used to generate a variegated population of AGS fragments for screening and subsequent selection of homologues of an AGS protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of an AGS coding sequence with a nuclease under conditions wherein nicking occurs only
10 about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-
15 terminal and internal fragments of various sizes of the AGS protein.

 Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of AGS
20 homologues. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was
25 detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify AGS homologues (Arkin and Yourvan (1992) *PNAS* 89:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

 An isolated AGS protein, or a portion or fragment thereof, can be used as an
30 immunogen to generate antibodies that bind AGS using standard techniques for polyclonal and monoclonal antibody preparation. The full-length AGS protein can be

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used or, alternatively, the invention provides antigenic peptide fragments of AGS for use as immunogens. The antigenic peptide of AGS comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of AGS such that an antibody raised against the peptide forms a specific immune complex with
5 AGS. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of AGS that are located on the surface of the protein, *e.g.*, hydrophilic regions and/or regions that are unique to AGS, *e.g.* not
10 common to all small G proteins.

An AGS immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed AGS protein or a chemically synthesized AGS peptide. The preparation can further
15 include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic AGS preparation induces a polyclonal anti- AGS antibody response.

Accordingly, another aspect of the invention pertains to anti- AGS antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and
20 immunologically active portions of immunoglobulin molecules, *e.g.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as AGS. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and
25 monoclonal antibodies that bind AGS. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of AGS. A monoclonal antibody composition thus typically displays a single binding affinity for a particular AGS protein with which it immunoreacts.

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Polyclonal anti- AGS antibodies can be prepared as described above by immunizing a suitable subject with an AGS immunogen. The anti- AGS antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized AGS. If
5 desired, the antibody molecules directed against AGS can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti- AGS antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard
10 techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown et al. (1981) *J. Immunol.* 127:539-46; Brown et al. (1980) *J. Biol. Chem.* 255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), the EBV-hybridoma
15 technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L.
20 Gefter et al. (1977) *Somatic Cell Genet.* 3:231-36).

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti- AGS monoclonal antibody (see, e.g., G. Galfre et al. (1977) *Nature* 266:55052; Gefter et al. *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth,
25 *Monoclonal Antibodies*, cited *supra*). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind AGS, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti- AGS antibody can be identified and isolated by screening a
30 recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with AGS to thereby isolate immunoglobulin library members that bind AGS.

Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. PCT International Publication No. WO 92/18619; Dower et al. PCT International Publication No. WO 91/17271; Winter et al. PCT International Publication WO 92/20791; Markland et al. PCT International Publication No. WO 92/15679; Breitling et al. PCT International Publication WO 93/01288; McCafferty et al. PCT International Publication No. WO 92/01047; Garrard et al. PCT International Publication No. WO 92/09690; Ladner et al. PCT International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J. Mol. Biol.* 226:889-896; Clarkson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas et al. (1991) *PNAS* 88:7978-7982; and McCafferty et al. *Nature* (1990) 348:552-554.

Additionally, recombinant anti- AGS antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson et al. International Application No. PCT/US86/02269; Akira, et al. European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al. European Patent Application 173,494; Neuberger et al. PCT International Publication No. WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al. European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-

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1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi et al. (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

5 An anti- AGS antibody (*e.g.*, monoclonal antibody) can be used to isolate AGS by standard techniques, such as affinity chromatography or immunoprecipitation. An anti- AGS antibody can facilitate the purification of natural AGS from cells and of recombinantly produced AGS expressed in host cells. Moreover, an anti- AGS antibody can be used to detect AGS protein (*e.g.*, in a cellular lysate or cell supernatant) in order
10 to evaluate the abundance, pattern of expression, and/or subcellular localization of the AGS protein. Anti- AGS antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*e.g.*, physically linking) the antibody to a detectable substance. Examples of detectable
15 substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include
20 umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

25

IV. Pharmaceutical Compositions

 The AGS nucleic acid molecules, AGS proteins, AGS modulators, and anti- AGS antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject,
30 *e.g.*, a human. Such compositions typically comprise the nucleic acid molecule, protein, modulator, or antibody and a pharmaceutically acceptable carrier. As used herein the

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language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid

polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various
5 antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for
10 example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., an AGS protein or anti- AGS antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating
15 the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered
20 solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared
25 using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as
30 microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant

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such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject

to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.* retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods of the Invention

An AGS protein of the invention has one or more of the activities described herein and can thus be used, for example, to screen drugs or compounds which modulate AGS protein activity as well as to treat disorders characterized by insufficient or excessive production of AGS protein or production of AGS protein forms which have altered (*e.g.*, increased or decreased) activity compared to wild type AGS. In addition, methods are provided that employ AGS molecules and rely on strategies based upon functional readouts using the yeast *Saccharomyces cerevisiae*, and these methods offer a fast and more reliable way to identify genes whose expression affects key aspects of cellular function. Accordingly, these methods have distinct advantages over transcriptional profiling screens, computer-based screens, screens based on protein-protein interactions, and other transgenic and cell-based functional screens that have been developed in higher eukaryotes (Spence, (1998) *Drug Disc. Today* 3:179-188;

Simonsen et al., (1994) *Trends Pharmacol. Sci.* 15:437-441; Evans et al., (1997) *Trends Genet.* 13:370-374; Hicks et al., (1997) *Nat. Genet.* 16:338-344; and Whitney et al., (1998) *Nat. Biotech.* 16:1329-1333). Moreover, the isolated nucleic acid molecules of the invention can be used to express AGS protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect AGS mRNA (*e.g.*, in a biological sample) or a genetic lesion in an AGS gene, and to modulate AGS activity, as described further below. Moreover, the anti- AGS antibodies of the invention can be used to detect and isolate AGS protein and modulate AGS protein activity.

10 a. Drug Screening Assays:

The invention provides methods for identifying compounds or agents which modulate AGS protein activity and/or AGS nucleic acid expression. These methods are also referred to herein as drug screening assays and typically include the step of screening a candidate/test compound or agent for the ability to interact with (*e.g.*, bind to) an AGS protein, to modulate the interaction of an AGS protein and a target molecule, and/or to modulate AGS nucleic acid expression and/or AGS protein activity, and/or to modulate signal transduction mediated at least in part by an AGS protein. Candidate/test compounds or agents which have one or more of these abilities can be used as drugs to treat disorders characterized by aberrant or abnormal AGS protein activity and/or AGS nucleic acid expression. Candidate/test compounds such as small molecules, *e.g.*, small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries.

In a preferred embodiment, the invention provides a method for identifying a compound that modulates signal transduction in a cell, comprising the steps of contacting a cell that expresses an AGS protein with a test compound and determining the effect of the test compound on the activity of the AGS protein and identifying the test compound as a modulator of signal transduction based on the ability of the compound to modulate the activity of the AGS protein in the cell.

As used herein, the term "identify" as used in the context of "identifying a compound" refers to the identification of compounds for which an activity as an activator of G protein signaling has not been previously recognized or demonstrated.

The term "identifying" according to the methods of the present invention is intended to refer to identifying, screening and/or selecting of test compound, for example selecting active compounds not previously recognized as activators of G protein signaling for further analysis and testing. In a preferred non-limiting example, compounds

5 "identified" according to the methods of the present invention can be used as test compounds in a second assay to confirm a G protein activating activity. Alternatively, compounds "identified" according to the methods of the present invention can be tested for other desirable activities or can be tested, for example, at varying doses to determine the efficacy of the compound. Compounds "identified" according to the methods of the

10 present invention can be also tested in cell culture models or in animal models of disease.

In a preferred embodiment, the AGS protein comprises an amino acid sequence having at least 86% identity to SEQ ID NO: 2 and stimulates G protein activity in a receptor-independent manner. In a particularly preferred embodiment, the AGS protein

15 comprises the amino acid sequence of SEQ ID NO 2. Alternatively, the AGS protein can comprise a structure as described above in the sections discussing AGS proteins and nucleic acids.

In a preferred embodiment, of the cell-based screening method, the cell has been engineered to express the AGS protein by introducing into the cell an expression vector

20 encoding the AGS protein. The cell can be further engineered to express other proteins, such as a G protein α subunit. In a preferred embodiment, the cell is a yeast cell that has been engineered to express an AGS protein and a mammalian or chimeric G protein α subunit and the effect of the test compound on the activity of the AGS protein is determined by monitoring a pheromone response pathway in the yeast cells. A preferred

25 chimeric G protein subunit which the yeast cell is engineered to express is a Gpa1-Gai2 chimeric G protein α subunit (preferably comprising 41 amino-terminal amino acids from yeast Gpa1 operatively linked to a mammalian Gai2). The pheromone response pathway in the yeast cells can be monitored, for example, by measuring the activity of a pheromone responsive promoter in the yeast cells. Yeast cell compositions and methods

that can be used for screening modulators of AGS proteins are described in further detail below and in the Examples.

In an alternative embodiment, the effect of the test compound on the activity of the AGS protein is determined by monitoring the ability of the test compound to bind to
5 the AGS protein.

In another alternative embodiment, the effect of the test compound on the activity of the AGS protein is determined by monitoring the ability of the test compound to modulate the interaction of the AGS protein with a target molecule. Preferably, the target molecule is a G protein.

10 In another embodiment, the invention provides assays for screening candidate/test compounds which interact with (*e.g.*, bind to) AGS protein. Typically, the assays are cell-free assays which include the steps of combining an AGS protein or a biologically active portion thereof, and a candidate/test compound, *e.g.*, under conditions which allow for interaction of (*e.g.*, binding of) the candidate/test compound to the AGS
15 protein or portion thereof to form a complex, and detecting the formation of a complex, in which the ability of the candidate compound to interact with (*e.g.*, bind to) the AGS protein or portion thereof is indicated by the presence of the candidate compound in the complex. Formation of complexes between the AGS protein and the candidate compound can be quantitated, for example, using standard immunoassays.

20 In another embodiment, the invention provides screening assays to identify candidate/test compounds which modulate (*e.g.*, stimulate or inhibit) the interaction (and most likely AGS activity as well) between an AGS protein and a molecule (target molecule) with which the AGS protein normally interacts. Examples of such target molecules includes proteins in the same signaling pathway as the AGS protein, *e.g.*, G
25 proteins, or proteins which may function upstream (including both stimulators and inhibitors of activity) or downstream of the G protein in the pheromone response pathway. Typically, the assays are cell-free assays which include the steps of combining an AGS protein or a biologically active portion thereof, an AGS target molecule (*e.g.*, a G protein) and a candidate/test compound, *e.g.*, under conditions wherein but for the
30 presence of the candidate compound, the AGS protein or biologically active portion thereof interacts with (*e.g.*, binds to) the target molecule, and detecting the formation of

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a complex which includes the AGS protein and the target molecule or detecting the interaction/reaction of the AGS protein and the target molecule. Detection of complex formation can include direct quantitation of the complex by, for example, measuring inductive effects of the AGS protein. A statistically significant change, such as a
5 decrease, in the interaction of the AGS and target molecule (*e.g.*, in the formation of a complex between the AGS and the target molecule) in the presence of a candidate compound (relative to what is detected in the absence of the candidate compound) is indicative of a modulation (*e.g.*, stimulation or inhibition) of the interaction between the AGS protein and the target molecule. Modulation of the formation of complexes
10 between the AGS protein and the target molecule can be quantitated using, for example, an immunoassay.

To perform the above drug screening assays, it may be desirable to immobilize either AGS or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate
15 automation of the assay. Interaction (*e.g.*, binding of) of AGS to a target molecule, in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For
20 example, glutathione-S-transferase/ AGS fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (*e.g.* ^{35}S -labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following
25 incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of AGS-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

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Other techniques for immobilizing proteins on matrices can also be used in the drug screening assays of the invention. For example, either AGS or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated AGS molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with AGS but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and AGS trapped in the wells by antibody conjugation. As described above, preparations of an AGS-binding protein and a candidate compound are incubated in the AGS-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the AGS target molecule, or which are reactive with AGS protein and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

The invention also provides cell-based assays for identifying compounds which modulate AGS activity. In one embodiment, the cell-based assay involves a method for identifying a compound which modulates (*e.g.*, stimulates or inhibits) AGS activity including contacting a cell which contains an AGS protein with a test compound and determining the ability of the test compound to modulate the activity of the AGS protein. In a preferred embodiment, determining the ability of the test compound to modulate AGS activity includes determining the ability of the test compound to effect (*e.g.*, upregulate or down regulate) G-protein mediated signaling in the cell. For example, G-protein mediated signaling can be determined in an AGS-containing cell prior to contacting the cell with a test compound and compared to the signaling after contacting the cell with a test compound. Compounds which reduce the G-protein mediated signaling can be identified as AGS antagonists whereas compounds which increase the G-protein mediated signaling can be identified as agonists.

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Alternatively, modulators of AGS expression can be identified in a method wherein a cell is contacted with a candidate compound and the expression of AGS mRNA or protein in the cell is determined. The level of expression of AGS mRNA or protein in the presence of the candidate compound is compared to the level of expression of AGS mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of AGS nucleic acid expression based on this comparison. For example, when expression of AGS mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of AGS mRNA or protein expression. Alternatively, when expression of AGS mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of AGS mRNA or protein expression. The level of AGS mRNA or protein expression in the cells can be determined by methods described herein for detecting AGS mRNA or protein.

The present invention also provides cell-based assays for identifying compounds which modulate the activity of an AGS. Typically, cells for use in cell based assays are engineered to express a heterologous AGS protein. In preferred embodiments, the cells may be further engineered such that the endogenous AGS protein is not expressed in functional form. Cells used to express a heterologous AGS can be, for example, mammalian or yeast in origin. In preferred embodiments, the engineered cells of the present invention are yeast cells.

In certain embodiments, the cells for use in the instant assays are further engineered to express a heterologous G protein coupled receptor which is functionally integrated into the signaling pathway of the cell in which it is expressed. In the case of yeast cells, heterologous GPCRs can be expressed in yeast cells and can be made to couple to yeast G proteins resulting in the transduction of signals via the endogenous yeast pheromone system signaling pathway which is normally activated by STE2 or STE3. In certain embodiments, such heterologous receptors can be made to couple more effectively to the yeast pheromone system signaling pathway by coexpressing a heterologous G protein subunit or subunits, by expressing a chimeric G protein subunit, or by expressing a chimeric G protein coupled receptor. Methods for preparing

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engineered yeast cells, and engineered yeast cells themselves, are described in U.S. Patent No. 5,482,835 by King et al. and PCT Publication WO 94/23025 by Fowlkes et al., the contents of both of which are hereby expressly incorporated herein by reference.

In one embodiment of the assay, the effect of a test compound on AGS induced
5 G protein activation can be measured by detecting changes in second messenger generation. Alternatively, in another embodiment, the effect of a test compound on an inhibitor of an activator of G-protein signaling can be detected. A variety of intracellular effectors have been identified as being G-protein-regulated, including adenylyl cyclase, cyclic GMP, phosphodiesterases, phosphoinositidase C, and phospholipase A₂. In
10 addition, G proteins interact with a range of ion channels and are able to inhibit certain voltage-sensitive Ca⁺⁺ transients, as well as stimulating cardiac K⁺ channels. For example, in yeast cells a reduction in the generation of AGS-induced second messengers or mating factor responses (e.g., growth arrest or shmoo formation) could be measured.

In one embodiment, GTP binding or GTPase enzymatic activity of G proteins
15 can be measured in plasma membrane preparations by determining, respectively, the incorporation of GTP γ ³⁵S or breakdown of γ ³²P GTP using techniques that are known in the art (For example, see *Signal Transduction: A Practical Approach*. G. Milligan, Ed. Oxford University Press, Oxford England). When receptors that modulate cAMP are tested, it will be possible to use standard techniques for cAMP detection, such as
20 competitive assays which quantitate [³H]cAMP in the presence of unlabelled cAMP.

Certain receptors stimulate the activity of phospholipase C which stimulates the breakdown of phosphatidylinositol 4,5, biphosphate to 1,4,5-IP₃ (which mobilizes intracellular Ca⁺⁺) and diacylglycerol (DAG) (which activates protein kinase C). Inositol lipids can be extracted and analyzed using standard lipid extraction techniques.
25 DAG can also be measured using thin-layer chromatography. Water soluble derivatives of all three inositol lipids (IP₁, IP₂, IP₃) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell can be measured using standard techniques. The choice of the appropriate
30 calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca⁺⁺-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the

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event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca^{++} detection, cells could be loaded with the Ca^{++} sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca^{++} measured using a fluorometer.

5 The other product of PIP2 breakdown, DAG can also be produced from phosphatidyl choline. The breakdown of this phospholipid in response to receptor-mediated signaling can also be measured using a variety of radiolabelling techniques.

 The activation of phospholipase A2 can easily be quantitated using known techniques, including, for example, the generation of arachadonate in the cell.

10 In the case of certain receptors, it may be desirable to screen for changes in cellular phosphorylation. Such assay formats may be useful when the receptor of interest is a receptor tyrosine kinase. For example, yeast transformed with the FGF receptor and a ligand which binds the FGF receptor could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430)
15 using anti-phosphotyrosine. In addition, tests for phosphorylation could be useful when a receptor which may not itself be a tyrosine kinase, activates protein kinases that function downstream in the signal transduction pathway. Likewise, it is noted that protein phosphorylation also plays a critical role in cascades that serve to amplify signals generated at the receptor. Multi-kinase cascades allow not only signal amplification but
20 also signal divergence to multiple effectors that are often cell-type specific, allowing a growth factor to stimulate mitosis of one cell and differentiation of another.

 One such cascade is the MAP kinase pathway that appears to mediate both mitogenic, differentiation and stress responses in different cell types. Stimulation of growth factor receptors results in Ras activation followed by the sequential activation of
25 c-Raf, MEK, and p44 and p42 MAP kinases (ERK1 and ERK2). Activated MAP kinase then phosphorylates many key regulatory proteins, including p90RSK and Elk-1 that are phosphorylated when MAP kinase translocates to the nucleus. Homologous pathways exist in mammalian and yeast cells. For instance, an essential part of the *S. cerevisiae* pheromone signaling pathway is comprised of a protein kinase cascade composed of the
30 products of the *STE11*, *STE7*, and *FUS3/KSS1* genes (the latter pair are distinct and functionally redundant). Accordingly, phosphorylation and/or activation of members of

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this kinase cascade can be detected and used to quantitate receptor engagement. Phosphotyrosine specific antibodies are available to measure increases in tyrosine phosphorylation and phospho-specific antibodies are commercially available (New England Biolabs, Beverly, MA).

5 Modified methods for detecting receptor-mediated signal transduction exist and one of skill in the art will recognize suitable methods that may be used to substitute for the example methods listed.

 In another embodiment, an indicator gene can be used for detection. In one embodiment an indicator gene is an unmodified endogenous gene. For example, in yeast
10 cells, the instant method can rely on detecting the transcriptional level of such pheromone system pathway responsive endogenous genes as the *BAR1* or *FUS1*, *FUS 2*, mating factor, *STE3* *STE13*, *KEX1*, *STE2*, *STE6*, *STE7*, *SST2*, or *CHS1*. (Appeltauer and Zchstetter. 1989. Eur. J. Biochem. 181:243)

 In other embodiments, the sensitivity of an endogenous indicator gene can be
15 enhanced by manipulating the promoter sequence at the natural locus for the indicator gene. Such manipulation may range from point mutations to the endogenous regulatory elements to gross replacement of all or substantial portions of the regulatory elements.

 For example, in the case of the *BAR1* gene, the promoter of the gene can be modified to enhance the transcription of *BAR1* upon activation of the yeast pheromone
20 system pathway. *BAR1* gene transcription is activated upon exposure of yeast cells to mating factor. The sequence of the *BAR1* gene is known in the art (see e.g., U.S. patent 4,613,572). Moreover, the sequences required for α -factor-enhanced expression of the Bar1, and other pheromone responsive genes have been identified. (Appeltauer and Achstetter 1989. Eur. J. Biochem. 181:243; Hagen et al. 1991. Mol. Cell. Biol.
25 11:2952). In an exemplary embodiment, the yeast *Bar1* promoter can be engineered by mutagenesis to be more responsive, e.g., to more strongly promote gene transcription, upon stimulation of the yeast pheromone pathway. Standard techniques for mutagenizing the promoter can be used. In such embodiments, it is desirable that the conserved oligonucleotide motif described by Appeltaure et al. be conserved.

In yet other embodiments, rather than measuring second messenger production or alterations in transcription, the activity of endogenous yeast proteins can be assayed. For example, in one embodiment, the signal transduction pathway of the receptor upregulates expression or otherwise activates an enzyme which is capable of modifying a substrate which can be added to the cell. The signal can be detected by using a detectable substrate, in which case loss of the substrate signal is monitored, or alternatively, by using a substrate which produces a detectable product. In certain embodiments, the substrate is naturally occurring. Alternatively, the substrate can be non-naturally occurring. In preferred embodiments, BAR1 activity can be measured.

In other embodiments, the modulation of a receptor by a test compound can result in a change in the transcription of a gene, which is not normally pheromone responsive. In preferred embodiments, the gene is easily detectable. For example, in a preferred embodiment, the subject assay can be used to measure Pho5, a secreted acid phosphatase. Acid phosphatase activity can be measured using standard techniques.

In other embodiments, reporter gene constructs can be used. Reporter gene constructs are prepared by operatively linking a reporter gene with at least one transcriptional regulatory element. If only one transcriptional regulatory element is included it must be a regulatable promoter. At least one of the selected transcriptional regulatory elements must be indirectly or directly regulated by the activity of the selected cell-surface receptor whereby activity of the receptor can be monitored via transcription of the reporter genes.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art. Reporter genes include any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties.

Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al.

(1987), *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin et al. (1984), *Biochemistry* 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) *Eur. J. Biochem.* 182: 231-238, Hall et al. (1983) *J. Mol. Appl. Gen.* 2: 101), human placental secreted alkaline phosphatase (Cullen and
5 Malim (1992) *Methods in Enzymol.* 216:362-368) and green fluorescent protein (U.S. patent 5,491,084; WO96/23898).

Transcriptional control elements include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose
10 expression is rapidly induced, generally within minutes, of contact between the cell surface protein and the effector protein that modulates the activity of the cell surface protein. Examples of such genes include, but are not limited to, the immediate early genes (see, Sheng et al. (1990) *Neuron* 4: 477-485), such as c-fos. Immediate early genes are genes that are rapidly induced upon binding of a ligand to a cell surface
15 protein. The transcriptional control elements that are preferred for use in the gene constructs include transcriptional control elements from immediate early genes, elements derived from other genes that exhibit some or all of the characteristics of the immediate early genes, or synthetic elements that are constructed such that genes in operative linkage therewith exhibit such characteristics. The characteristics of preferred genes
20 from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular stimulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life.
25 It is not necessary for all of these properties to be present.

Other promoters and transcriptional control elements, in addition to those described above, include the vasoactive intestinal peptide (VIP) gene promoter (cAMP responsive; Fink et al. (1988), *Proc. Natl. Acad. Sci.* 85:6662-6666); the somatostatin gene promoter (cAMP responsive; Montminy et al. (1986), *Proc. Natl. Acad. Sci.*
30 83:6682-6686); the proenkephalin promoter (responsive to cAMP, nicotinic agonists, and phorbol esters; Comb et al. (1986), *Nature* 323:353-356); the phosphoenolpyruvate

carboxy-kinase gene promoter (cAMP responsive; Short et al. (1986), J. Biol. Chem. 261:9721-9726); the NGFI-A gene promoter (responsive to NGF, cAMP, and serum; Changelian et al. (1989). Proc. Natl. Acad. Sci. 86:377-381); and others that may be known to or prepared by those of skill in the art.

5 In certain assays it may be desirable to use changes in growth in the screening procedure. For example, one of the consequences of activation of the pheromone signal pathway in wild-type yeast is growth arrest. If one is testing for an antagonist of a G protein-coupled receptor, this normal response of growth arrest can be used to select cells in which the pheromone response pathway is inhibited. That is, cells exposed to
10 both a known agonist and a peptide of unknown activity will be growth arrested if the peptide is neutral or an agonist, but will grow normally if the peptide is an antagonist. Thus, the growth arrest response can be used to advantage to discover peptides that function as antagonists.

 However, when searching for compounds which can function as modulators of
15 pheromone response pathways, the growth arrest consequent to activation of the pheromone response pathway is an undesirable effect since cells that bind agonists stop growing while surrounding cells that fail to bind agonists will continue to grow. The cells of interest, then, will be overgrown or their detection obscured by the background cells, confounding identification of the cells of interest. To overcome this problem the
20 present invention teaches engineering the cell such that: 1) growth arrest does not occur as a result of exogenous signal pathway activation (*e.g.*, by inactivating the FAR1 gene); and/or 2) a selective growth advantage is conferred by activating the pathway (*e.g.*, by transforming an auxotrophic mutant with a HIS3 gene under the control of a pheromone-responsive promoter, and applying selective conditions).

25 Alternatively, the promoter may be one which is repressed by the receptor pathway, thereby preventing expression of a product which is deleterious to the cell. With a receptor repressed promoter, one screens for agonists by linking the promoter to a deleterious gene, and for antagonists, by linking it to a beneficial gene. Repression may be achieved by operably linking a receptor-induced promoter to a gene encoding mRNA
30 which is antisense to at least a portion of the mRNA encoded by the marker gene (whether in the coding or flanking regions), so as to inhibit translation of that mRNA.

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Repression may also be obtained by linking a receptor-induced promoter to a gene encoding a DNA binding repressor protein, and incorporating a suitable operator site into the promoter or other suitable region of the marker gene.

In the case of yeast, exemplary positively selectable (beneficial) genes include the following: *URA3*, *LYS2*, *HIS3*, *LEU2*, *TRP1*; *ADE1*, 2, 3, 4, 5, 7, 8; *ARG1*, 3, 4, 5, 6, 8; *HIS1*, 4, 5; *ILV1*, 2, 5; *THR1*, 4; *TRP2*, 3, 4, 5; *LEU1*, 4; *MET2*, 3, 4, 8, 9, 14, 16, 19; *URA1*, 2, 4, 5, 10; *HOM3*, 6; *ASP3*; *CHO1*; *ARO* 2, 7; *CYS3*; *OLE1*; *INO1*, 2, 4; *PRO1*, 3. Countless other genes are potential selective markers. The above are involved in well-characterized biosynthetic pathways. The imidazoleglycerol phosphate dehydratase (IGP dehydratase) gene (*HIS3*) is preferred because it is both quite sensitive and can be selected over a broad range of expression levels. In the simplest case, the cell is auxotrophic for histidine (requires histidine for growth) in the absence of activation. Activation leads to synthesis of the enzyme and the cell becomes prototrophic for histidine (does not require histidine). Thus the selection is for growth in the absence of histidine. Since only a few molecules per cell of IGP dehydratase are required for histidine prototrophy, the assay is very sensitive.

In another embodiment a reporter gene, *e.g.*, the *fus1-lacZ* reporter plasmid can be introduced along with the AGS. The addition of an antagonist should result in a decrease in β -galactosidase units over that observed in the absence of the antagonist, demonstrating the ability of the antagonist to interact in a negative fashion with the AGS.

In another version of the assay, cells can be selected for resistance to aminotriazole (AT), a drug that inhibits the activity of IGP dehydratase. Cells with low, fixed level of expression of *HIS3* are sensitive to the drug, while cells with higher levels are resistant. The amount of AT can be selected to inhibit cells with a basal level of *HIS3* expression (whatever that level is) but allow growth of cells with an induced level of expression. In this case selection is for growth in the absence of histidine and in the presence of a suitable level of AT.

In appropriate assays, so-called counterselectable or negatively selectable genes may be used. Suitable genes include: *URA3* (orotidine-5'-phosphate decarboxylase; inhibits growth on 5-fluoroorotic acid), *LYS2* (2-aminoadipate reductase; inhibits

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growth on α -amino adipate as sole nitrogen source), CYH2 (encodes ribosomal protein L29; cycloheximide-sensitive allele is dominant to resistant allele), CAN1 (encodes arginine permease; null allele confers resistance to the arginine analog canavanine), and other recessive drug-resistant markers. *CAN1* is one example of a preferred reporter gene.

In one example, the reporter gene affects yeast cell growth. The natural response to signal transduction via the yeast pheromone system response pathway is for cells to undergo growth arrest. This is a preferred way to select for antagonists of a ligand/receptor pair that stimulates a the pathway. An antagonist would inhibit the activation of the pathway; hence, the cell would be able to grow. Thus, the FAR1 gene may be considered an endogenous counterselectable marker. The FAR1 gene is preferably inactivated when screening for agonist activity.

The reporter gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include beta-galactosidase (Xgal, C₁₂FDG, Salmon-gal, Magenta-Gal (latter two from Biosynth Ag)), alkaline phosphatase, horseradish peroxidase, exo-glucanase (product of yeast *exb1* gene; nonessential, secreted); luciferase; bacterial green fluorescent protein; (human placental) secreted alkaline phosphatase (SEAP); and chloramphenicol transferase (CAT). Some of the above can be engineered so that they are secreted (although not β -galactosidase). A preferred screenable marker gene is beta-galactosidase; yeast cells expressing the enzyme convert the colorless substrate Xgal into a blue pigment. Again, the promoter may be receptor-induced or receptor-inhibited.

In another example using yeast cells, a screen can take advantage of the fact that a *gpa1 fus1 -HIS3* colony expressing wild type G α s can grow upon replica plating to media lacking histidine and containing 1mM 3-aminotriazole (AT). The growth of this strain occurs due to the partially constitutive state of the pheromone pathway, which leads to partial derepression of the *fus1-HIS3* reporter gene. AT inhibits the activity of IGP dehydratase. Cells with low, fixed level of expression of HIS3 are sensitive to the drug, while cells with higher levels are resistant. The amount of AT can be selected to

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inhibit cells with a basal level of HIS3 expression (whatever that level is) but allow growth of cells with an induced level of expression. A colony to which an antagonist of an AGS has been added will presumably fail to grow on this media due to the reduced signaling via the pheromone pathway.

5 In certain embodiments, a peptide library can be expressed to test for modulators of an AGS. In one embodiment, the peptide library is expressed by the cell that also expresses the AGS, thereby creating an "autocrine" system, wherein the peptide to be tested for AGS-modulating activity is made by the same cell expressing the AGS protein. For a general description of yeast cell autocrine systems for screening peptide, 10 see PCT Publication WO 94/23025 by Fowlkes et al., the contents of which are expressly incorporated herein by reference. In certain embodiments in which yeast cells are used, such a library can be expressed using a leader sequence for periplasmic expression, *e.g.*, a yeast mating factor leader sequence. Yeast cells are bounded by a lipid bilayer called the plasma membrane. Between this plasma membrane and the cell 15 wall is the periplasmic space. Peptides secreted by yeast cells cross the plasma membrane through a variety of mechanisms and thereby enter the periplasmic space. The secreted peptides are then free to interact with other molecules that are present in the periplasm or displayed on the outer surface of the plasma membrane. The peptides then either undergo re-uptake into the cell, diffuse through the cell wall into the medium, or 20 become degraded within the periplasmic space.

The test polypeptide library may be secreted into the periplasm by any of a number of exemplary mechanisms, depending on the nature of the expression system to which they are linked. In one embodiment, the peptide may be structurally linked to a yeast signal sequence, such as that present in the α -factor precursor, which directs 25 secretion through the endoplasmic reticulum and Golgi apparatus.

An alternative mechanism for delivering peptides to the periplasmic space is to use the ATP-dependent transporters of the STE6/MDR1 class. This transport pathway and the signals that direct a protein or peptide to this pathway are not as well characterized as is the endoplasmic reticulum-based secretory pathway. Nonetheless, 30 these transporters apparently can efficiently export certain peptides directly across the plasma membrane, without the peptides having to transit the ER/Golgi pathway. It is

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anticipated that at least a subset of peptides can be secreted through this pathway by expressing the library in context of the α -factor prosequence and terminal tetrapeptide. Use of either of the described pathways is within the scope of the invention.

The present invention does not require periplasmic secretion of peptides, or, if
5 such secretion is provided, any particular secretion signal or transport pathway. In certain embodiments, peptides expressed with a signal sequence may bind to and modulate AGS molecules prior to their transport to the cell surface.

In other embodiments, a heterologous G-protein coupled receptor can be coexpressed with a heterologous AGS protein. In embodiments in which yeast cells are
10 used, it may be desirable to use the leader sequence of a yeast secreted protein to direct transport of G-protein coupled receptors to the plasma membrane.

In certain embodiments, the compounds to be tested in the subject assays can be derived from libraries. While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures
15 of other compounds, such as benzodiazepines (Bunin et al. 1992. J. Am. Chem. Soc. 114:10987; DeWitt et al. 1993. Proc. Natl. Acad. Sci. USA 90:6909) peptoids (Zuckermann. 1994. J. Med. Chem. 37:2678) oligocarbamates (Cho et al. 1993. Science. 261:1303), and hydantoins (DeWitt et al. supra). Rebek et al. have described an approach for the synthesis of molecular libraries of small organic molecules with a
20 diversity of 10⁴-10⁵ (Carell et al. 1994. Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al. Angew. Chem. Int. Ed. Engl. 1994. 33:2061).

The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries,
25 synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. Anticancer Drug Des. 1997. 12:145).

In one embodiment, the test compound is a peptide or peptidomimetic. In another, preferred embodiment, the compounds are small, organic non-peptidic compounds.

Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: Erb et al. 1994. *Proc. Natl. Acad. Sci. USA* 91:11422; Horwell et al. 1996 *Immunopharmacology* 33:68; and in Gallop et al. 1994. *J. Med. Chem.* 37:1233. In addition, libraries such as those described in the commonly owned applications U.S.S.N. 08/864,241, U.S.S.N. 08/864,240 and U.S.S.N. 08/835,623 can be used to provide compounds for testing in the present invention. The contents of each of these applications is expressly incorporated herein by this reference.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In certain embodiments, the test compounds are exogenously added to the yeast cells expressing a recombinant receptor and compounds that modulate signal transduction via the receptor are selected. In other embodiments, the yeast cells express the compounds to be tested. For example, a culture of the subject yeast cells can be further modified to collectively express a peptide library as described in more detail in PCT Publication WO 94/23025 the contents of which is expressly incorporated herein by this reference.

Other types of peptide libraries may also be expressed, see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/ 02502). In still another embodiment, the combinatorial polypeptides are produced from a cDNA library.

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries. In such embodiments, both compounds which agonize or

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antagonize the receptor- or channel-mediated signaling function can be selected and identified.

In certain other embodiments, cells can be engineered to produce the compounds to be tested. This assay system has the advantage of increasing the effective
5 concentration of the compound to be tested. In one embodiment, a method such as that described in WO 94/23025 can be utilized.

Other methods can also be used. For example, peptide libraries are systems which simultaneously display, in a form which permits interaction with a target, a highly diverse and numerous collection of peptides. These peptides may be presented in
10 solution (Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-
15 6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*). Many of these systems are limited in terms of the maximum length of the peptide or the composition of the peptide (*e.g.*, Cys excluded). Steric factors, such as the proximity of a support, may interfere with binding. Usually, the screening is for binding *in vitro* to an artificially presented target, not for activation or inhibition of a cellular signal transduction pathway
20 in a living cell. While a cell surface receptor may be used as a target, the screening will not reveal whether the binding of the peptide caused an allosteric change in the conformation of the receptor.

The Ladner et al. patent, USSN 5,096,815, describes a method of identifying novel proteins or polypeptides with a desired DNA binding activity. Semi-random
25 ("variegated") DNA encoding a large number of different potential binding proteins is introduced, in expressible form, into suitable yeast cells. The target DNA sequence is incorporated into a genetically engineered operon such that the binding of the protein or polypeptide will prevent expression of a gene product that is deleterious to the gene under selective conditions. Cells which survive the selective conditions are thus cells
30 which express a protein which binds the target DNA. While it is taught that yeast cells may be used for testing, bacterial cells are preferred. The interactions between the

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protein and the target DNA occur only in the cell (and then only in the nucleus), not in the periplasm or cytoplasm, and the target is a nucleic acid, and not a receptor protein. Substitution of random peptide sequences for functional domains in cellular proteins permits some determination of the specific sequence requirements for the accomplishment of function. Though the details of the recognition phenomena which operate in the localization of proteins within cells remain largely unknown, the constraints on sequence variation of mitochondrial targeting sequences and protein secretion signal sequences have been elucidated using random peptides (Lemire et al., *J. Biol. Chem.* (1989) 264, 20206 and Kaiser et al. (1987) *Science* 235:312, respectively).

10 In certain embodiments of the instant invention, the compounds tested are in the form of peptides from a peptide library. The peptide library of the present invention takes the form of a cell culture, in which essentially each cell expresses one, and usually only one, peptide of the library. While the diversity of the library is maximized if each cell produces a peptide of a different sequence, it is usually prudent to construct the library so there is some redundancy. Depending on size, the combinatorial peptides of the library can be expressed as is, or can be incorporated into larger fusion proteins. The fusion protein can provide, for example, stability against degradation or denaturation, as well as a secretion signal if secreted. In an exemplary embodiment of a library for intracellular expression, e.g., for use in conjunction with intracellular target receptors,

15 the polypeptide library is expressed as thioredoxin fusion proteins (see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/ 02502). The combinatorial peptide can be attached one the terminus of the thioredoxin protein, or, for short peptide libraries, inserted into the so-called active loop.

In one embodiment, the peptide library is derived to express a combinatorial library of polypeptides which are not based on any known sequence, nor derived from cDNA. That is, the sequences of the library are largely random. In preferred embodiments, the combinatorial polypeptides are in the range of 3-100 amino acids in length, more preferably at least 5-50, and even more preferably at least 10, 13, 15, 20 or 25 amino acid residues in length. Preferably, the polypeptides of the library are of

25 uniform length. It will be understood that the length of the combinatorial peptide does

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not reflect any extraneous sequences which may be present in order to facilitate expression, *e.g.*, such as signal sequences or invariant portions of a fusion protein.

In another embodiment, the peptide library is a combinatorial library of polypeptides which are based at least in part on a known polypeptide sequence or a portion thereof (not a cDNA library). That is, the sequences of the library is semi-random, being derived by combinatorial mutagenesis of a known sequence. See, for example, Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461. Accordingly, polypeptide(s) which are known ligands for a target receptor can be mutagenized by standard techniques to derive a variegated library of polypeptide sequences which can further be screened for agonists and/or antagonists.

In still another embodiment, the combinatorial polypeptides are produced from a cDNA library.

In a preferred embodiment of the present invention, the cells collectively produce a "peptide library", preferably including at least 10^3 to 10^7 different peptides, so that diverse peptides may be simultaneously assayed for the ability to interact with the exogenous receptor. In an especially preferred embodiment, at least some peptides of the peptide library are secreted into the periplasm, where they may interact with the "extracellular" binding site(s) of an exogenous receptor. They thus mimic more closely the clinical interaction of drugs with cellular receptors. This embodiment optionally may be further improved (in assays not requiring pheromone secretion) by preventing pheromone secretion, and thereby avoiding competition between the peptide and the pheromone for signal peptidase and other components of the secretion system.

In certain embodiments of the present invention, the peptides of the library are encoded by a mixture of DNA molecules of different sequence. Each peptide-encoding DNA molecule is ligated with a vector DNA molecule and the resulting recombinant DNA molecule is introduced into a yeast cell. Since it is a matter of chance which peptide encoding DNA molecule is introduced into a particular cell, it is not predictable which peptide that cell will produce. However, based on a knowledge of the manner in

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which the mixture was prepared, one may make certain statistical predictions about the mixture of peptides in the peptide library.

The peptides of the library can be composed of constant and variable residues. If the *n*th residue is the same for all peptides of the library, it is said to be constant. If the *n*th residue varies, depending on the peptide in question, the residue is a variable one. The peptides of the library will have at least one, and usually more than one, variable residue. A variable residue may vary among any of two to all twenty of the genetically encoded amino acids; the variable residues of the peptide may vary in the same or different manner. Moreover, the frequency of occurrence of the allowed amino acids at a particular residue position may be the same or different. The peptide may also have one or more constant residues.

There are two principal ways in which to prepare the required DNA mixture. In one method, the DNAs are synthesized a base at a time. When variation is desired, at a base position dictated by the Genetic Code, a suitable mixture of nucleotides is reacted with the nascent DNA, rather than the pure nucleotide reagent of conventional polynucleotide synthesis.

The second method provides more exact control over the amino acid variation. First, trinucleotide reagents are prepared, each trinucleotide being a codon of one (and only one) of the amino acids to be featured in the peptide library. When a particular variable residue is to be synthesized, a mixture is made of the appropriate trinucleotides and reacted with the nascent DNA. Once the necessary "degenerate" DNA is complete, it must be joined with the DNA sequences necessary to assure the expression of the peptide, as discussed in more detail below, and the complete DNA construct must be introduced into the yeast cell.

In the case of yeast cells, test polypeptide library may be secreted into the periplasm by any of a number of exemplary mechanisms, depending on the nature of the expression system to which they are linked. In one embodiment, the peptide may be structurally linked to a yeast signal sequence, such as that present in the α -factor precursor, which directs secretion through the endoplasmic reticulum and Golgi apparatus. Since this is the same route that the receptor protein follows in its journey to the plasma membrane, opportunity exists in cells expressing both the receptor and the

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peptide library for a specific peptide to interact with the receptor during transit through the secretory pathway. This has been postulated to occur in mammalian cells exhibiting autocrine activation. Such interaction could yield activation of the response pathway during transit, which would still allow identification of those cells expressing a peptide agonist. For situations in which peptide antagonists to externally applied receptor agonist are sought, this system would still be effective, since both the peptide antagonist and receptor would be delivered to the outside of the cell in concert. Thus, those cells producing an antagonist would be selectable, since the peptide antagonist would be properly and timely situated to prevent the receptor from being stimulated by the externally applied agonist.

An alternative mechanism for delivering peptides to the periplasmic space is to use the ATP-dependent transporters of the STE6/MDR1 class. This transport pathway and the signals that direct a protein or peptide to this pathway are not as well characterized as is the endoplasmic reticulum-based secretory pathway. Nonetheless, these transporters apparently can efficiently export certain peptides directly across the plasma membrane, without the peptides having to transit the ER/Golgi pathway. It is anticipated that at least a subset of peptides can be secreted through this pathway by expressing the library in context of the a-factor prosequence and terminal tetrapeptide. The possible advantage of this system is that the receptor and peptide do not come into contact until both are delivered to the external surface of the cell. Thus, this system strictly mimics the situation of an agonist or antagonist that is normally delivered from outside the cell. Use of either of the described pathways is within the scope of the invention. The present invention does not require periplasmic secretion, or, if such secretion is provided, any particular secretion signal or transport pathway.

After identifying certain test compounds as potential modulators of AGS proteins, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

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The compounds selected in the subject assay, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof.

- 5 The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active
- 10 substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing
- 15 Company, Easton, Pa., USA 1985).

- In yet another aspect of the invention, the AGS proteins can be used as "bait proteins" in a two-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and
- 20 Brent WO94/10300), to identify other proteins, which bind to or interact with AGS ("AGS-binding proteins" or "AGS-bp") and modulate AGS protein activity. Such AGS-binding proteins are also likely to be involved in the propagation of signals by the AGS proteins as, for example, upstream or downstream elements of the pheromone response pathway.

- 25 The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, ("bait"), the gene that codes for AGS, is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a
- 30 library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If

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the "bait" and the "prey" proteins are able to interact, *in vivo*, forming an AGS-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with AGS.

Another aspect of the invention is directed to a yeast-based counter-screen for pheromone pathway inhibitors. This screen is based on a yeast cell constitutively expressing a receptor-independent activator (*e.g.*, AGS1) of the yeast pheromone response pathway such that a lethal marker gene is expressed (*e.g.*, Can1). Then, a cDNA library of interest, cloned into an appropriate vector (*e.g.*, pYES2), is introduced into this strain to identify cDNAs that, when expressed, counteract the activators ability to induce the expression of the lethal marker gene. This screen, therefore, can rapidly identify proteins that both directly and indirectly regulate activators of the pheromone response pathway.

Modulators of AGS protein activity and/or AGS nucleic acid expression identified according to these drug screening assays can be used to treat, for example, diseases or disorders characterized by excessive or insufficient G-protein mediated signal transduction. Examples of such diseases or disorders which can be treated using modulators of AGS protein activity and/or nucleic acid expression include proliferative disorders and/or diseases. Support for the use of AGS modulators in treating proliferative disorders can be found in the fact that, for example, oncogenic mutations in ras-like G proteins have been implicated in approximately 30% of human cancers, including 90% of pancreatic and 50% of colon cancers (Bos (1988) *Mutat. Res.* 195:255-271; Bos (1989) *Cancer Res.* 49:4682-4689). In addition, defects in GPCR-mediated signaling have been associated with various disease states (Landis et al. (1989) *Nature* 340:692-696 (pituitary tumors); Lyons et al. (1990) *Science* 249:655-659 (human endocrine tumors); Allen et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:11354-11358 (neoplastic transformation and athlerosclerosis); and Farfel et al. (1996) *J. Biol. Chem.* 271:19653-19655 (pseudohypoparathyroidism)). As AGS is both a member of the ras

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family and an activator of heterotrimeric G-protein mediated signal transduction, modulators of AGS function are likely to have pharmaceutical applications. Methods of treatment include the steps of administering the AGS molecules of the present invention or modulators of AGS protein activity and/or nucleic acid expression, *e.g.*, in a pharmaceutical composition as described above, to a subject in need of such treatment.

b. Detection and Diagnostic Assays:

The invention further provides a method for detecting the presence of AGS in a biological sample. The method involves contacting the biological sample with a compound or an agent capable of detecting AGS protein or mRNA such that the presence of AGS is detected in the biological sample. A preferred agent for detecting AGS mRNA is a labeled or labelable nucleic acid probe capable of hybridizing to AGS mRNA. The nucleic acid probe can be, for example, the AGS cDNA of SEQ ID NO: 1, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to AGS mRNA. A preferred agent for detecting AGS protein is a labeled or labelable antibody capable of binding to AGS protein. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')₂) can be used. The term "labeled or labelable", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*e.g.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect AGS mRNA or protein in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of AGS mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of AGS protein include enzyme linked immunosorbent assays (ELISAs),

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Western blots, immunoprecipitations and immunofluorescence. Alternatively, AGS protein can be detected *in vivo* in a subject by introducing into the subject a labeled anti-AGS antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one preferred embodiment of the detection method, the biological sample is a cell sample, a tissue section, for example, a freeze-dried or fresh frozen section of tissue removed from a patient, or a biological fluid obtained from a subject.

The invention also encompasses kits for detecting the presence of AGS in a biological sample. For example, the kit can comprise a labeled or labelable compound or agent capable of detecting AGS protein or mRNA in a biological sample; means for determining the amount of AGS in the sample; and means for comparing the amount of AGS in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect AGS mRNA or protein.

c. Methods of Treatment:

Additional methods of the invention include methods for treating a subject having a disorder characterized by aberrant AGS activity or expression. These methods include administering to the subject an AGS modulator such that treatment of the subject occurs. The terms "treating" or "treatment", as used herein, refer to reduction or alleviation of at least one adverse effect or symptom of a disease or disorder, *e.g.*, a disease or disorder characterized by or associated with abnormal or aberrant AGS protein activity or AGS nucleic acid expression.

As used herein, an AGS modulator is a molecule which can modulate AGS nucleic acid expression and/or AGS protein activity. For example, an AGS modulator can modulate, *e.g.*, upregulate (activate) or downregulate (suppress), AGS nucleic acid expression. In another example, an AGS modulator can modulate (*e.g.*, stimulate or inhibit) AGS protein activity. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) AGS nucleic acid expression and/or AGS protein activity by inhibiting AGS nucleic acid expression, an

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AGS modulator can be an antisense molecule, *e.g.*, a ribozyme, as described herein. Examples of antisense molecules which can be used to inhibit AGS nucleic acid expression include antisense molecules which are complementary to a portion of the 5' untranslated region of SEQ ID NO: 3, which also includes the start codon and antisense molecules which are complementary to a portion of the 3' untranslated region of SEQ ID NO:3. AN AGS modulator which inhibits AGS nucleic acid expression can also be a small molecule or other drug, *e.g.*, a small molecule or drug identified using the screening assays described herein, which inhibits AGS nucleic acid expression. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) AGS nucleic acid expression and/or AGS protein activity by stimulating AGS nucleic acid expression, an AGS modulator can be, for example, a nucleic acid molecule encoding AGS (*e.g.*, a nucleic acid molecule comprising a nucleotide sequence homologous to the nucleotide sequence of SEQ ID NO:1) or a small molecule or other drug, *e.g.*, a small molecule (peptide) or drug identified using the screening assays described herein, which stimulates AGS nucleic acid expression.

Alternatively, if it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) AGS nucleic acid expression and/or AGS protein activity by inhibiting AGS protein activity, an AGS modulator can be an anti- AGS antibody or a small molecule or other drug, *e.g.*, a small molecule or drug identified using the screening assays described herein, which inhibits AGS protein activity. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) AGS nucleic acid expression and/or AGS protein activity by stimulating AGS protein activity, an AGS modulator can be an active AGS protein or portion thereof (*e.g.*, an AGS protein or portion thereof having an amino acid sequence which is homologous to the amino acid sequence of SEQ ID NO:2) or a small molecule or other drug, *e.g.*, a small molecule or drug identified using the screening assays described herein, which stimulates AGS protein activity.

In another embodiment, an AGS modulator is an indirect modulator (*e.g.*, an indirect activator or inhibitor of AGS protein expression and/or activity. For example, it may be possible to modulate the expression of an AGS protein by targeting a transcription factor which activates or represses AGS gene transcription. Accordingly,

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an AGS modulator is an indirect modulator which increases the expression and/or activity of an AGS-specific transcription factor. In another embodiment, an AGS modulator is an indirect modulator which decreases the expression and/or activity of an AGS-specific transcription factor.

- 5 Other aspects of the invention pertain to methods for modulating a cell associated activity. These methods include contacting the cell with an agent (or a composition which includes an effective amount of an agent) which modulates AGS activity or AGS expression such that a cell associated activity is altered relative to a cell associated activity of the cell in the absence of the agent. As used herein, "a cell associated
- 10 activity" refers to a normal or abnormal activity or function of a cell. Examples of cell associated activities include proliferation, migration, differentiation, production or secretion of molecules, such as proteins, and cell survival. In a preferred embodiment, the cell-associated activity is mediated by a G-protein signaling pathway. The term "altered" or "modulated" as used herein refers to a change, *e.g.*, an increase or decrease,
- 15 of a cell associated activity. In one embodiment, the agent stimulates AGS protein activity or AGS nucleic acid expression. Examples of such stimulatory agents include an active AGS protein, a nucleic acid molecule encoding AGS that has been introduced into the cell, and a modulatory agent which stimulates AGS protein activity or AGS nucleic acid expression and which is identified using the drug screening assays described
- 20 herein. In another embodiment, the agent inhibits AGS protein activity or AGS nucleic acid expression. Examples of such inhibitory agents include an antisense AGS nucleic acid molecule, an anti- AGS antibody, and a modulatory agent which inhibits AGS protein activity or AGS nucleic acid expression and which is identified using the drug screening assays described herein. These modulatory methods can be performed *in vitro*
- 25 (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). In a preferred embodiment, the modulatory methods are performed *in vivo*, *e.g.*, the cell is present within a subject, *e.g.*, a mammal, *e.g.*, a human, and the subject has a disorder or disease characterized by or associated with abnormal or aberrant AGS activity or expression.

A nucleic acid molecule, a protein, an AGS modulator etc. used in the methods of treatment can be incorporated into an appropriate pharmaceutical composition described herein and administered to the subject through a route which allows the molecule, protein, modulator etc. to perform its intended function. Examples of routes of administration are also described herein. Particular AGS inhibitory agents and AGS stimulatory agents are described further below.

Inhibitory Agents

According to a modulatory method of the invention, AGS activity is inhibited in a cell by contacting the cell with an inhibitory agent. Inhibitory agents of the invention can be, for example, intracellular binding molecules that act to inhibit the expression or activity of AGS. As used herein, the term "intracellular binding molecule" is intended to include molecules that act intracellularly to inhibit the expression or activity of a protein by binding to the protein itself, to a nucleic acid (*e.g.*, an mRNA molecule) that encodes the protein or to a target with which the protein indirectly interacts. Examples of intracellular binding molecules, described in further detail below, include polypeptides that directly or indirectly bind an AGS molecule or target molecule, antisense AGS nucleic acid molecules (*e.g.*, to inhibit translation of AGS mRNA), intracellular anti-AGS antibodies (*e.g.*, to inhibit the activity of AGS protein) and chemical inhibitors of the AGS protein.

In one embodiment, an inhibitor of an AGS or AGS-related molecule is identified by modifying one of the above-mentioned assays to activate a reporter gene that prevents growth (*e.g.*, *CAN1*). Thus, candidate inhibitors of an AGS or AGS-related activator that can block the AGS induction of the growth inhibiting gene can be rapidly screened and identified (Fig. 2b). This variation of the above assay may be used for either the screening of a nucleic acid library or a compound library. Accordingly, in one embodiment, an identified inhibitor of an AGS molecule is the RGS5 polypeptide (SEQ ID NO: 25) which either directly or indirectly down-regulates AGS activity.

In another embodiment, an inhibitory agent of the invention is an antisense nucleic acid molecule that is complementary to a gene encoding AGS or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a

particular protein in a cell is well known in the art (see *e.g.*, Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986; Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R. W. (1994) *Nature* 372:333-335). *e.g.e.g.* Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated region of an mRNA. An antisense nucleic acid for inhibiting the expression of AGS protein in a cell can be designed based upon the nucleotide sequence encoding the AGS protein (*e.g.*, SEQ ID NO: 1), constructed according to the rules of Watson and Crick base pairing (*e.g.*, as described above in subsection I).

An antisense nucleic acid can exist in a variety of different forms. For example, the antisense nucleic acid can be an oligonucleotide that is complementary to only a portion of an AGS gene. An antisense oligonucleotides can be constructed using chemical synthesis procedures known in the art. *e.g.* To inhibit AGS expression in cells in culture, one or more antisense oligonucleotides can be added to cells in culture media, typically at about 200 µg oligonucleotide/ml.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*e.g.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). *e.g.*

In another embodiment, an antisense nucleic acid for use as an inhibitory agent is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region (for reviews on ribozymes see *e.g.*, Ohkawa, J. *et al.* (1995) *J. Biochem.* 118:251-258; Sigurdsson, S.T. and Eckstein, F. (1995) *Trends Biotechnol.* 13:286-289; Rossi, J.J. (1995) *Trends Biotechnol.* 13:301-306; Kiehnopf, M. *et al.* (1995) *J. Mol. Med.* 73:65-71). A ribozyme having specificity for AGS mRNA can be designed based upon the nucleotide sequence of the AGS cDNA. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base

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sequence of the active site is complementary to the base sequence to be cleaved in an AGS mRNA. See for example U.S. Patent Nos. 4,987,071 and 5,116,742, both by Cech *et al.* Alternatively, AGS mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and
5 Szostak, J.W. (1993) *Science* 261: 1411-1418.

Another type of inhibitory agent that can be used to inhibit the expression and/or activity of AGS in a cell is an intracellular antibody specific for the AGS protein. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-
15 5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

20 To inhibit protein activity using an intracellular antibody, a recombinant expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell. For inhibition of AGS activity according to the inhibitory methods of the invention, an intracellular antibody
25 that specifically binds the AGS protein is expressed in the cytoplasm of the cell. To prepare an intracellular antibody expression vector, antibody light and heavy chain cDNAs encoding antibody chains specific for the target protein of interest, *e.g.*, AGS, are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for the AGS protein. Hybridomas secreting anti-AGS monoclonal antibodies, or
30 recombinant anti-AGS monoclonal antibodies, can be prepared as described above. Once a monoclonal antibody specific for AGS protein has been identified (*e.g.*, either a

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hybridoma-derived monoclonal antibody or a recombinant antibody from a combinatorial library), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR
5 amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from the display package (*e.g.*, phage) isolated during the library screening process. Nucleotide sequences of antibody light and heavy chain genes from which PCR primers or cDNA library probes can be prepared are known in the art. For example, many such
10 sequences are disclosed in Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database.

Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods. To allow for cytoplasmic
15 expression of the light and heavy chains, the nucleotide sequences encoding the hydrophobic leaders of the light and heavy chains are removed. An intracellular antibody expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes full-length antibody light and heavy chains such that a full-length antibody is expressed
20 intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed intracellularly. In the most preferred embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible peptide linker (*e.g.*, (Gly₄Ser)₃) and expressed as a single chain molecule. To
25 inhibit AGS activity in a cell, the expression vector encoding the anti-AGS intracellular antibody is introduced into the cell by standard transfection methods, as discussed hereinbefore.

Other inhibitory agents that can be used to inhibit the activity of an AGS protein are chemical compounds that directly inhibit AGS activity or inhibit the interaction
30 between AGS and target molecules. Such compounds can be identified using screening assays that select for such compounds, as described in detail above.

Stimulatory Agents

According to a modulatory method of the invention, AGS activity is stimulated in a cell by contacting the cell with a stimulatory agent. Examples of such stimulatory agents include active AGS protein and nucleic acid molecules encoding AGS that are introduced into the cell to increase AGS activity in the cell. A preferred stimulatory agent is a nucleic acid molecule encoding an AGS protein, wherein the nucleic acid molecule is introduced into the cell in a form suitable for expression of the active AGS protein in the cell. To express an AGS protein in a cell, typically an AGS-encoding DNA is first introduced into a recombinant expression vector using standard molecular biology techniques, as described herein. AN AGS-encoding DNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR), using primers based on the AGS nucleotide sequence. Following isolation or amplification of AGS-encoding DNA, the DNA fragment is introduced into an expression vector and transfected into target cells by standard methods, as described herein.

Other stimulatory agents that can be used to stimulate the activity of an AGS protein are chemical compounds that stimulate AGS activity in cells, such as compounds that directly stimulate AGS protein and compounds that promote the interaction between AGS and target molecules. Such compounds can be identified using screening assays that select for such compounds, as described in detail above.

The modulatory methods of the invention can be performed *in vitro* (e.g., by culturing the cell with the agent or by introducing the agent into cells in culture) or, alternatively, *in vivo* (e.g., by administering the agent to a subject or by introducing the agent into cells of a subject, such as by gene therapy). For practicing the modulatory method *in vitro*, cells can be obtained from a subject by standard methods and incubated (e.g., cultured) *in vitro* with a modulatory agent of the invention to modulate AGS activity in the cells. If desired, cells treated *in vitro* with a modulatory agent of the invention can be readministered to the subject. For administration to a subject, it may be preferable to first remove residual agents in the culture from the cells before administering them to the subject. For further discussion of *ex vivo* genetic modification of cells followed by readministration to a subject, see also U.S. Patent No. 5,399,346 by W.F. Anderson *et al.*

For practicing the modulatory method *in vivo* in a subject, the modulatory agent can be administered to the subject such that AGS activity in cells of the subject is modulated. The term "subject" is intended to include living organisms in which an AGS-dependent cellular response can be elicited. Preferred subjects are mammals.

5 Examples of subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep.

For stimulatory or inhibitory agents that comprise nucleic acids (including recombinant expression vectors encoding AGS protein, antisense RNA and intracellular antibodies), the agents can be introduced into cells of the subject using methods known
10 in the art for introducing nucleic acid (*e.g.*, DNA) into cells *in vivo*. Examples of such methods encompass both non-viral and viral methods, including:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly injecting the DNA into the cells (see *e.g.*, Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (*e.g.*, a
15 "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (*e.g.*, from BioRad).

Cationic Lipids: Naked DNA can be introduced into cells *in vivo* by complexing the DNA with cationic lipids or encapsulating the DNA in cationic liposomes. Examples of suitable cationic lipid formulations include N-[-1-(2,3-
20 dioleoyloxy)propyl]N,N,N-triethylammonium chloride (DOTMA) and a 1:1 molar ratio of 1,2-dimyristyloxy-propyl-3-dimethylhydroxyethylammonium bromide (DMRIE) and dioleoyl phosphatidylethanolamine (DOPE) (see *e.g.*, Logan, J.J. *et al.* (1995) *Gene Therapy* 2:38-49; San, H. *et al.* (1993) *Human Gene Therapy* 4:781-788).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells
25 *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus
30 capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes

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(see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271).

- 5 A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant
- 10 retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψ Crip, ψ Cre, ψ 2
- 15 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018;
- 20 Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991) *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu
- 25 *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid
- 30 into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 *etc.*) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka *et al.* *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985)

Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (*e.g.*, Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product.

In a preferred embodiment, a retroviral expression vector encoding AGS is used to express AGS protein in cells *in vivo*, to thereby stimulate AGS protein activity *in vivo*. Such retroviral vectors can be prepared according to standard methods known in the art (discussed further above).

A modulatory agent, such as a chemical compound, can be administered to a subject as a pharmaceutical composition. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. Pharmaceutical compositions can be prepared as described above.

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This invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLES

5

The following experimental procedures were used in the Examples:

Plasmids and yeast strains - All DNA manipulations were done using standard recombinant DNA techniques (see *e.g.*, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; and Ausubel et al. (1994) *Current Protocols in Molecular Biology*, John Wiley & Sons, NY); using commercially available enzymes (New England Biolabs, BRL, United States Biochemical Corporation). Transformations of bacterial strain DH10B were performed by electroporation at 1.8 kV in a GenePulser II (BioRad). Transformations of yeast were performed as described in Ito et al. (1983) *J. Bacteriol.* 153:163-168; and Elble, R. (1992) *Biotechniques* 13:18-19.

15

Yeast strains used in this study are listed below in Table 1.

TABLE 1:

20

<u>YEAST STRAIN</u>	<u>GENOTYPE</u>
CY1316	<i>MATα gpa1Δ far1Δ tbt1-1 fus1p-HIS3 can1 ste14::trp1::LYS2 ste3Δ lys2 ura3 leu2 trp1 his3</i>
CY4600	<i>MATα gpa1Δ ste4Δ far1Δ tbt1-1 fus1p-HIS3 can1 ste14::trp1::LYS2 ste3Δ lys2 ura3 leu2 trp1 his3</i>
CY12444	<i>MATα gpa1Δ ste5Δ far1Δ tbt1-1</i>

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		<i>fus1p-HIS3 can1 ste14::trp1::LYS2</i>
		<i>ste3Δ lys2 ura3 leu2 trp1 his3</i>
	CY12970	<i>MATα gpa1Δ ste20Δ far1Δ fus1p-</i>
		<i>HIS3 can1 ste14::trp1::LYS2 ste3Δ</i>
5		<i>lys2 ura3 leu2 trp1 his3</i>
	CY1141	<i>MATα GPA1(1-41)-Gai2 far1Δ</i>
		<i>tbt1-1 fus1p-HIS3 can1</i>
		<i>ste14::trp1::LYS2 ste3Δ lys2 ura3</i>
		<i>leu2 trp1 his3</i>
10	CY7967	<i>MATα GPA1(1-41)-Gai3 far1Δ</i>
		<i>tbt1-1 fus1p-HIS3 can1</i>
		<i>ste14::trp1::LYS2 ste3Δ lys2 ura3</i>
		<i>leu2 trp1 his3</i>
	CY8342	<i>MATα [rat Gas] far1Δ tbt1-1</i>
15		<i>fus1p-HIS3 can1 ste14::trp1::LYS2</i>
		<i>ste3Δ lys2 ura3 leu2 trp1 his3</i>
	CY9603	<i>MATα GPA1(1-41)-Gal6(S270P)</i>
		<i>far1Δ tbt1-1 fus1p-HIS3 can1</i>
		<i>ste14::trp1::LYS2 ste3Δ lys2 ura3</i>
20		<i>leu2 trp1 his3</i>
	CY9571	<i>MATα GPA1 far1Δ tbt1-1 fus1p-</i>
		<i>HIS3 can1 ste14::trp1::LYS2 ste3Δ</i>
		<i>lys2 ura3 leu2 trp1 his3</i>

CY1316 (*MAT α gal Δ far Δ tbt1-1 fus1-HIS3 can1 ste14::trp1::LYS2 ste3 Δ lys2 ura3 leu2 trp1 his3*), the parent of all strains used in this study, was obtained by standard genetic techniques, with SY1390 (Stevenson et al. (1992) *Genes Dev.* 6:1293-1304) (provided by G. Sprague), and SM1188 (Sapperstein et al. (1994) *Mol. Cell. Biol.* 14:1438-1449) (provided by S. Michaelis) serving as the original sources of the *fus1-HIS3* and *ste14* alleles, respectively. Unless otherwise indicated, all genomic disruptions were made with the *URA3* gene, followed by selection on 5'-fluoroorotic acid (Boeke et al. (1987) *Methods. Enz.* 154:164-195). $G\alpha$ genomic integrations were made at the *GPA1* locus and verified by Southern, $G\alpha$ expression, and phenotypic analysis. Plasmid pR15 (Beals et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7886-7890), carrying the coding region of human $G\alpha i2$. Plasmid CP1127, carrying the promoter sequences and first 41 amino acid codons of *GPA1*, was prepared by ligation of a sequence encompassing nucleotides -200 to +100 of *GPA1* (where translational start is +1) to pRS405 (Sikorski and Hieter (1989) *Genetics* 122:19). Plasmid CP1183, carrying the *GPA1*(1-41)- $G\alpha i2$ chimera sequence, was made by PCR amplification of the $G\alpha i2$ coding region encompassing amino acids 36 to its stop codon at position 357 using the oligo pair SEQ ID NO: 4 and SEQ ID NO: 5 and using plasmid pR15 as template. The amplified product was digested with *SacI* and *SalI*, then ligated into *SacI/SalI* digested CP1127. A glycine to alanine alteration at codon 204 of $G\alpha i2$ in CP1183 was introduced using Stratagene's QuickChange kit and mutagenic oligos SEQ ID NO: 6 and SEQ ID NO: 7 creating plasmid CP5533. Sequences encoding β -galactosidase (*lacZ*) were introduced downstream of the *fus1* promoter on plasmid pRS424 (Sikorski and Hieter (1989) *Genetics* 122:19) to create CP1584. Plasmid pSM187, with a 4.3 kb DNA fragment carrying the *STE14* gene flanked by *Bam*HI sites, was kindly provided by S. Michaelis. This *Bam*HI fragment was inserted into *Bam*HI digested and shrimp alkaline phosphatase treated pRS415 and pRS414 (Sikorski and Hieter (1989) *Genetics* 122:19) to create, respectively, plasmids CP5108 and CP5336.

The yeast phosphoglycerate kinase (*PGKI*) promoter sequence was amplified from yeast genomic DNA using the oligonucleotide pair SEQ ID NOs: 26 and 27, digested with *Bgl*II and *Nco*I, and ligated into *Esp*3I/*Nco*I digested Yep51Nco (Broach

et al. (1983) *In Experimental Manipulation of Gene Expression* (M. Inouye, ed.) Academic Press, NY). A 0.95 kb fragment encompassing this promoter was then excised with *Bgl*II and *Bam*HI for ligation into pRS415 and pRS424 (Sikorski and Hieter (1989) *Genetics* 122:19). The coding sequence for human RGS4 was amplified by reverse-transcriptase PCR from human brain poly-A selected RNA and ligated downstream of the *PGK*I promoter in pRS424. RGS4 sequences were then amplified by PCR using the oligonucleotide pair SEQ ID NOS: 28 and 29, digested with *Bam*HI and *Xho*I, and ligated into *Bam*HI/*Xho*I digested pYES2 (Invitrogen). *FUS*1 promoter sequences were amplified from yeast genomic DNA using the oligonucleotide pair SEQ ID NOS: 30 and 31, digested with *Kpn*I and *Sal*I, and gel purified. The *CAN*1 coding region was amplified from yeast genomic DNA using the oligonucleotide pair SEQ ID NOS: 32 and 33, digested with *Xho*I and *Pst*I, and gel purified. Both purified fragments were ligated to *Pst*I/*Kpn*I digested pRS414 (Sikorski et al. (1989) *Genetics* 122:19-27) to create plasmid 2440. The coding sequence for human AGS1 (see below) was amplified by PCR using the oligonucleotide pair SEQ ID NOS: 34 and 35, digested with *Bsp*HI and *Sal*I, and ligated into a *Nco*I/*Sal*I digested derivative of pRS415 carrying the *PGK*I promoter, creating plasmid 1451-AGS1. The Stratagene QuikChange kit and protocol were used to introduce a glycine to valine alteration at codon 31 of AGS1 in pYES2 using the mutagenic oligonucleotides of SEQ ID NOS: 36 and 37, as well as a cysteine to serine alteration at codon 278 of AGS1 using the mutagenic oligonucleotides of SEQ ID NOS: 38 and 39.

A customized adult human liver cDNA library, ligated into the yeast vector pYES2 at *Bst*XI and *Eco*RI, was constructed by Invitrogen. This library was size selected at 1250 bp and contains approximately 1.77×10^7 primary recombinants.

The vector pYEX4Ti, which allows for copper inducible expression in yeast of sequences fused at the N-terminus with GST, was purchased from Amrad Biotech. Vector pGEX-KG, which allows for IPTG inducible expression in bacteria of sequences fused at the N-terminus with GST (Guan and Dixon (1991), *Anal. Biochem.* 192, 262-267). Similar vectors are also commercially available. The vectors pcDNA3.1HisC and pBlueBacHis2A, which allow for expression of N-terminal hexahistidine fusion proteins, were purchased from Invitrogen. The vector pIND which allows for inducible

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expression from mammalian cells, was purchased from Invitrogen. The cDNA for gene AGS was amplified from pYES2-AGS by PCR using the oligo pair provided in SEQ ID NOS: 8 and 9, cleaved with *Bam*HI and *Eco*RI, and ligated into *Bam*HI/*Eco*RI digested pYEX4Ti, pGEX-KG, pcDNA3.1HisC, pBlueBacHis2A and pIND. The coding
5 sequence for Cdc42 was amplified by PCR from yeast genomic DNA using the oligo pair provided in SEQ ID NOS: 10 and 11, cleaved with *Bam*HI and *Eco*RI, and ligated into *Bam*HI/*Eco*RI digested pGEX-KG. Single glycine to valine alterations at codons 31 and 36, and a glycine to alanine alteration at codon 81, were constructed using the Stratagene QuickChange kit and protocol and the respective mutagenic oligo pairs
10 provided in SEQ ID NOS: 12 and 13, 14 and 15, and 16 and 17. Additional amino acid substitutions, using appropriately designed oligonucleotides and standard techniques, were also introduced into AGS1 at the following residue positions (in brackets): serine to glycine (33); serine to alanine (80); asparagine to glycine (82); phenylalanine to asparagine (44); serine to glycine (33) and serine to alanine (80); serine
15 to glycine (33) and asparagine to glycine (82); serine to alanine (80) and asparagine to glycine (82); serine to glycine (33), serine to alanine (80), and asparagine to glycine (82); serine to glycine (33), serine to alanine (80), asparagine to glycine (82), and glycine to alanine (81); lysine to glutamic acid (225) and lysine to glutamic acid (226); and, cysteine to serine (278). The plasmid pcDNA3.1HisC-AGS served as a template
20 for mutagenesis. Following mutagenesis, AGS sequences were excised with *Bam* HI and *Eco* RI for subcloning into vectors described above. *Bam* HI and *Eco* RI sites were introduced via PCR, for example the *Bam* HI site was introduced using the primer set forth as SEQ ID NO:8 and the *Eco* RI site, introduced using the primer set forth as SEQ ID NO:9. Automated dideoxy sequencing was used to verify the correct construction of
25 all plasmids used in the examples.

Yeast screens - All yeast strains were grown in synthetic medium (0.67% yeast nitrogen base without amino acids supplemented with amino acids, adenine, uracil and 2% carbon source as indicated). For the activator screens, cultures of CY1316/CP1183 were grown in glucose-supplemented medium lacking tryptophan to a cell density of
30 approximately 2×10^7 /ml prior to transformation with lithium acetate. Approximately 20 µg of an adult human liver cDNA library in vector pYES2 was used to transform $1 \times$

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10⁹ cells. Transformation mixtures were plated onto sucrose medium lacking tryptophan and uracil and grown at 30° for 20-22 hr. Replicas of each plate were then made onto galactose medium lacking tryptophan, uracil and histidine, and containing 1 mM AT. Replica plates were incubated at 30° for 72 hr and growing colonies patched
5 onto sucrose plates lacking tryptophan and uracil for recovery. Cells from each patch were resuspended in sterile water and equal numbers of cells (approximately 2,000) were spotted onto 3 plates: sucrose medium lacking tryptophan and uracil; glucose medium lacking tryptophan, uracil and histidine and containing 1 mM AT; and galactose medium lacking tryptophan, uracil and histidine and containing 1 mM AT. Plates were grown at
10 30° for 48 hr. Isolates with a galactose-dependent growth phenotype were cultured in liquid glucose medium lacking tryptophan and uracil for plasmid recovery.

For the inhibitor screen, initial transformations of CY1141/2440/1451-AGS1 were performed as described above for the activator screen. Replicas of each plate were made onto selective galactose medium lacking uracil and arginine and containing 200
15 µg/ml canavanine. Replica plates were incubated at 30° for 72 hr and growing colonies patched onto selective sucrose plates lacking uracil for recovery. Cells from each patch were resuspended in sterile water and spotted as described above onto selective sucrose medium lacking uracil, and onto selective glucose or galactose medium lacking uracil and arginine and containing 200 µg/ml canavanine. Plates were grown at 30° for 24 hr
20 then replica-plated onto identical medium and allowed to grow for an additional 24 hr to distinguish differential growth on galactose. Isolates with a galactose-dependent growth phenotype were cultured in liquid glucose medium lacking uracil for plasmid recovery. Strains CY1141/1451/2440 and CY1141/1451-AGS1/2440 were analyzed in a similar fashion on selective glucose medium lacking histidine and containing 2 mM AT or on
25 selective glucose medium lacking arginine and containing 200 µg/ml canavanine.

Plasmids were isolated from yeast essentially as described in Strathern and Higgins (1991) *Methods Enz.* 194:319-329, except for the omission of the affinity purification step, and transformed by electroporation into DH10B for amplification and re-transformation into yeast. Isolated plasmids were digested with *Bst*XI and *Eco*RI and
30 electrophoresed on 1% agarose gels for analysis of insert size. Fresh cultures of yeast

strains CY1316/CP1183 or CY1141/2440/1451-AGS1 were transformed with each isolated plasmid, plated onto sucrose medium lacking tryptophan and uracil, and grown at 30° for 20-22 hr. Replica plating and spotting assays were then performed as described above to identify plasmids that conferred a galactose-dependent growth phenotype on all transformants. Sequence of inserts was determined by automated dideoxy sequencing using primers homologous to pYES2 vector sequences in the T7 promoter region and the *CYC1* terminator region, as well as primers internal to the insert sequence.

Epistasis tests - Epistasis analysis on yeast strains was performed by introducing the pYES2 vector alone or AGS carried on the pYES2 vector into the indicated strains. Strains were grown to saturation in liquid sensitive sucrose medium. Approximately 2000 cells from each transformant were spotted onto selective sucrose medium (to determine viability) and selective glucose and galactose medium lacking histidine and containing 1mM AT. Plates were incubated at 30° for 2 days prior to photographing.

β-galactosidase assays - Indicated yeast strains carrying a plasmid-borne *FUS1-lacZ* reporter construct were grown for 16-18 hours in selective glucose or galactose medium. Cells were harvested in log phase. Aliquots of cells (100 μl) were placed in 96-well microtiter plates and 20μl of 6X CPRG-Z buffer (350 mM Na₂HPO₄, 240 mM NaH₂PO₄, 60mM KCl, 6mM MgSO₄, 2.5% Triton X-100, 16ul/ml β-mercaptoethanol, 10mM (chlorophenol red-β-D-galactopyranoside (CPRG) was added. Plates were incubated at 37° and reactions stopped with 60μl 1M sodium carbonate. Plates were read at 575 nm (using a Beckman Biomek® plate reader) and lacZ activity determined using the equation 1000 x A₅₇₅/ (incubation time in min) (OD₆₀₀ of original culture. All assays were done in triplicate on three independent isolates of each strain, and average activity +/- SEM was calculated. Data were analyzed using GraphPad Prism® software (Version 2.01).

Sequence Alignments - Sequence alignments were performed using the CLUSTAL X multiple sequence alignment program (Version 1.0). CLUSTAL X is a windows-based version of CLUSTAL V (Higgins et al. (1991) *CABIOS* 8:189-191), modified as described in Thompson et al. (1994) *Nuc. Acids Res.* 22:4673-4680.

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Alternatively, polynucleotide alignments were performed using BLASTN (version 2.0.8; see <http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi>) using the following parameters: match, 1; mismatch, -2; gap open, 5; gap extension, 2; x_dropoff, 50; expect, 10.0; wordsize, 11; and, filter, on. When aligning polypeptide sequence, BLASTP was
5 employed (see above web address) using the following parameters: matrix, O BLOSUM 62; gap open, 11; gap extension, 1; x_dropoff, 50; expect, 10.0; wordsize, 3; and, filter, on.

Analysis of tissue-specific expression of AGS - The full length coding sequence of AGS was generated by digestion of pYEX4Ti-AGS with *Bam* HI and *Eco* RI,
10 followed by two rounds of agarose gel purification (Qiagen). This fragment was radiolabelled with α -[32 P]-deoxycytidine triphosphate using the *red*i-prime kit and protocol (Amersham). Radiolabelled DNA was purified over a Sephadex G-50 spin column (Boehringer Mannheim). The radiolabelled fragment (1.46×10^9 cpm/ μ g) was hybridized in a volume of 20 ml (1.2×10^6 cpm/ml) to Multiple Tissue Northern Blots
15 from human tissues and human cancer cell lines (Clontech #7750-1, #7757-1, #7760-1, #7754-1, and #7765-1) and to a Human RNA Master Blot (Clontech #7770-1) using the protocols provided by Clontech. Filters were exposed to Fuji X-ray film at -80° with intensifying screens prior to development.

Cell transfections - HEK293 cells were grown in Dulbecco's minimal essential
20 medium (DMEM), supplemented with 10% fetal bovine serum and 1X penicillin/streptomycin, at 37° in a humidified atmosphere containing 5% CO₂. Cells were fed every 2 d and split at least once per week.

One day prior to transfection, cells were seeded at a density of 8×10^5 per 100-mm dish. Cells were transfected with 10 μ g of pcDNA3.1-HisC vector (Invitrogen, San
25 Diego, CA) or pcDNA3.1-HisC-AGS using SuperFect transfection reagent (Qiagen, Santa Clarita, CA) and the protocol provided by the manufacturer. Transfected cells were selected for resistance to the antibiotic Neomycin (0.5 μ g/ml). Selection was begun 3 d after transfection and continued for two weeks. Constitutive expression of AGS (from the CMV promoter in pcDNA3.1-HisC) was monitored in Neomycin

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resistant clones by RNA blot analysis using the full length cDNA coding sequence of AGS as probe (labeled with α -[^{32}P]-deoxycytidine triphosphate as described above).

Cultures of *Spodoptera frugiperda* (Sf9) were transfected with recombinant pBlueBacHisA-AGS. AGS-positive transfectants were screened for the production of β -galactosidase and AGS-positive phage were amplified according to the protocols
5 provided by Invitrogen.

In vitro transcription/translation assay - Protein expression from the original pYES2-AGS isolate and the pcDNA3.1-HisC-AGS construct was determined by coupled *in vitro* transcription/translation reactions using the kit, protocol, and control
10 plasmid from Clontech. Protein was labeled with ^{35}S -methionine by standard techniques and final products electrophoresed on a 12% SDS-polyacrylamide gel. Following electrophoresis, protein was electroblotted onto a PVDF membrane; the blot was washed extensively with water, air dried, and exposed without screens to Fuji X-ray film for 24 hr at ambient temperature.

Analysis of AGS protein expression levels - Expression levels of AGS tagged at the N-terminus with either GST or the His6 epitope were determined. For expression in *Saccharomyces cerevisiae* strain CY1141 and *E. coli* strain BL21, AGS was introduced with an N-terminal GST fusion under control of the copper-inducible *cup1* promoter on plasmid pYEX4Ti-AGS (yeast) or an IPTG-inducible P_{tac} promoter on plasmid pGEX-
20 KG-AGS (bacteria). Plasmid CP5336, carrying an episomal copy of *STE14*, was also introduced into the yeast strains. For copper induction, cells were grown at 30° to a density of 5×10^6 cells/ml and treated with 0.1 mM CuSO_4 for 6 hr. Cells were harvested at a density of $1-2 \times 10^7$ /ml, and cell extracts were prepared by bead lysis in the presence of denaturation buffer (240 mM Tris-HCl, pH 6.8, 20% glycerol, 2% (w/v)
25 SDS, 0.5M β -mercaptoethanol), followed by denaturation at 100° for 5 min. For IPTG induction, cells were grown at 30° in 2xYT supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin to an initial optical density at 600 nm of 0.5, then treated with 0.5 mM IPTG for 3 hr. Cells were harvested by centrifugation and whole cell extracts prepared by denaturation at 100° for 5 min in denaturation buffer.

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For transient expression in insect cells, recombinant phage carrying the pBlueBacHisA-AGS construct were used to infect cultures of Sf9 cells. 2-3 d post-infection, cells were harvested by centrifugation and whole cell extracts prepared by denaturation at 100° for 5 min in denaturation buffer. Stable transfectants of HEK293
5 cells carrying pcDNA3.1-HisC-AGS were cultured to ~80% confluency in DMEM and harvested by scraping in the presence of PBS. Cells were lysed at 4° in 5 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA containing a protease inhibitor cocktail (Boehringer Mannheim Complete™) by mechanical disruption using a glass Dounce tissue grinder (Wheaton, Millville, NJ). Following a low-speed centrifugation (3000 x g, 10 min, 4°)
10 to remove unlysed cells, supernatants were centrifuged at 100,000 x g, 30 min, 4° to separate membrane and soluble fractions. Membrane pellets were resuspended by brief sonication in lysis buffer and protein fractions denatured at 100° for 5 min in denaturation buffer.

Proteins were electrophoresed on 11% SDS-polyacrylamide gels (Laemmli
15 (1970) *Nature* 227:680-685) and electroblotted onto PVDF membranes. Membranes were blocked in 5% dry milk (w/v) in TBS-T and probed with either a 1:2000 dilution of antiserum raised against GST from *Schistosoma japonicum* (Sigma), a 1:2000 dilution of monoclonal anti-RGSHis6 antiserum (Qiagen), a 1:2000 dilution of monoclonal anti-His C-terminal antiserum (Invitrogen), or a 1:5000 dilution of anti-Xpress antiserum
20 (Invitrogen). Blots were washed extensively with TBS-T and probed with a 1:10000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse antiserum (Amersham) for 1 hr at 25°. After washing with TBS-T, blots were developed using the chemiluminescent substrate and protocol from Pierce.

Preparing Membrane Fractions - For determining protein localization, membrane
25 fractions from mammalian cells, (e.g., CHO-K1 transiently transfected (72 hr post-transfection) were scraped from 150 mm dishes, washed twice in PBS, and cell pellets lysed by 10-20 strokes of a Dounce homogenizer in 5 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, protease inhibitor cocktail, at 4° Lysates were centrifuged at 100,000 x g for 30 min at 4° C, supernatant fractions removed, and crude membrane pellets resuspended

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by Dounce homogenization in 50 mM Tris, pH 7.4, 0.6 mM EDTA, 5 mM MgCl₂, and protease inhibitors were added prior to immunoblot analysis.

Antisera Specific for AGS1 Polyclonal antisera specific for the AGS1 protein were raised in rabbit against the peptide sequence DTKSCLKNKTKENVD (SEQ. ID NO. 42) (amino acids 123 through 137 of AGS1) through Cocalico Biologicals®. These antisera were determined to recognize AGS1 protein expressed in bacteria and mammalian cells by standard immunoblot analysis.

Immunoblot Analysis- Yeast cell extracts, other cell extracts, and membrane fractions were immunoblotted using standard techniques and (the chemiluminescent substrate and protocol from Pierce.

Analysis of Gpal(1-41)-Gαi2 expression levels – Yeast strains CY1316/CP1183 and CY1316/CP5533 containing either pYES2 or pYES2-AGS were grown for 20 hr at 30° in medium containing 2% galactose and lacking tryptophan and uracil to a final OD₆₀₀ of approximately 1. Cells were harvested by centrifugation, washed once with H₂O, and lysed at 4° by mechanical disruption using 0.5 mm glass beads in 5 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA containing a protease inhibitor cocktail (Boehringer Mannheim Complete™). Following a low-speed centrifugation (3000 x g, 4° C, 10 min) to remove cell debris, samples were centrifuged at 100,000 x g, 4° C, 30 min to pellet crude membrane. Membrane pellets were resuspended in lysis buffer by brief sonication and samples were denatured, electrophoresed on 11% SDS-polyacrylamide gels and analyzed by immunoblotting as described above. Antiserum specific for the C-terminal region of Gαi2 (DuPont NEN) was used at a dilution of 1:1000.

Purification of tagged AGS for biochemical analysis - GST-AGS and GST-Cdc42 were purified from IPTG-induced *E. coli* cells carrying pGEX-KG-AGS or pGEX-KG-CDC42 using standard techniques. GST, GST-AGS, and GST-Cdc42 were purified from copper-induced yeast cells carrying pYEX-4T1, pYEX-4T1-AGS, or pYEX-4T1-Cdc42.

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Interaction assays - GST and an N-terminal GST fusion of AGS1 were expressed in yeast from the *CUP1* promoter on plasmid pYEX-4T1 (Armad) and bound to glutathione sepharose matrix (Pharmacia). After extensive washing, aliquots of matrix with bound GST fusion proteins were mixed with 100 μ l extracts from Sf9 cells expressing either His₆-Gai2 or the Ste4/His₆-Ste18 dimer for 1 hour at 4°. Extracts from His₆-Gai2 expressing cells were preincubated with either 1mM GDP or 1mM GTP γ S for 45 minutes at 25° C prior to incubation with GST affinity matrices. Samples were washed extensively and bound protein eluted at 100° C for 5 minutes in denaturation buffer. Equivalent amounts of eluted protein were analyzed by immunoblotting using a 1:5000 dilution of anti-Xpress antisera (Invitrogen; recognized His₆ fusion epitope) or a 1:1000 dilution of anti-Ste4 antisera. Bound antibody was detected by chemiluminescence (Pierce).

Kinase Assays - The p42/p44 MAP kinase assays were performed using the enzyme assay system (Amersham Life Science BIOTRAK® code RPN 84) essentially as according to the manufacturer. Briefly, cells (*e.g.*, EcR-CHO-K1, obtained from 15 Invitrogen) were transiently transfected with pIND/LacZ or pIND/AGS1 DNA constructs, EcR-3T3 (obtained from Invitrogen) cells transiently transfected with pIND/LacZ or pIND/AGS1 DNA constructs, or CHO-K1 cells stably expressing the human Nociceptin receptor (CADUS clone #14), were induced to produce LacZ or 20 AGS1 expression using with 5 μ M Ponasterone A for 5 and 16 hours. Cells were either pretreated with Pertusis toxin (50 ng/ml for 18 hours.) and/or stimulated with serum (3%) or nociceptin (100 nM).

After stimulation cells were washed, lysed, exposed to Magnesium [³²P] ATP (in appropriate buffers), and incubated for 30 min at 37° C. Reactions were then stopped 25 with a stop reagent (10 μ l), centrifuge for 15 sec at 14,000 x g, aliquoted (30 μ l) on to binding paper, washed, and counts were recorded as a measure of kinase activity.

Biochemical Analysis of Purified GST-AGS1 - AGS1 were induced from cells carrying, respectively, plasmid pYEX4T1 or pYEX4T1-AGS1 with 0.2 mM CuSO₄ at 30° for 6 hr. Induced cells were harvested by centrifugation, washed in PBS, and lysed 30 by vortexing with glass beads (0.5 mm; Biospec) in lysis buffer (50 mM Tris-HCl, pH

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7.6, 50 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 5 mM MgSO₄, 1 µl/ml Antifoam A (Sigma®). All buffers used were at 4° and contained protease inhibitors (Boehringer Mannheim Complete™, EDTA-free). GST and GST lysates were centrifuged at 10,000 x g at 4°C for 15 min and supernatants mixed batch-wise on a rotating platform with glutathione sepharose (Pharmacia) for 2 hr at 4° C. Samples were washed with 20 column volumes 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.01% Thesit, 5 mM MgSO₄, and eluted with 3 column volumes 50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 5 mM MgSO₄, 0.01% Thesit, 20 mM reduced glutathione. The His₆-Gai2 and Ste4/His₆-Ste18 dimer were purified from infected Sf9 cells after lysis in 110 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.5% Triton-X100, 10 µM GDP by gentle rocking at 4° for 30 min, followed by centrifugation at 10,000 rpm for 10 min. Lysates were bound batch-wise to a nickel-agarose matrix (ProBond; Invitrogen®) for 1 hr at 4°. Samples were washed sequentially with 10 column volumes lysis buffer, 10 column volumes low pH buffer (20 mM NaPO₄, pH 6.0, 500 mM NaCl, 5 mM MgCl₂, 10 µM GDP, 0.01% Thesit, 50 mM imidazole), 10 column volumes neutralization buffer (50 mM Tris-HCl, pH 7.0, 250 mM NaCl, 5 mM MgCl₂, 0.01% Thesit), and eluted with 3 column volumes neutralization buffer containing 300 mM imidazole.

All purified proteins were dialyzed against 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgSO₄, 0.01% Thesit, 1 mM DTT and quick-frozen in dry ice for storage. Protein purity was monitored by gel electrophoresis and immunoblotting as described herein. Gα protein concentration was determined by saturation binding with excess radiolabelled GTPαS; GST fusion protein concentrations were determined by a dye-binding assay (BioRad®).

Protein Association Assays - Purified Gai2 and Gβγ (0.3 µM) were mixed with either purified GST or GST-AGS1 (1.5 µM) in 100 µl 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 0.6 mM EGTA, 0.01% Thesit for 45 min at 25°. Gαi2 (at 7 µM) was allowed to equilibrate with 1 mM GDP or 1 mM guanosine 5'-O-(3-thiotriphosphate) (GTPαS) for 45 min at 25° prior to dilution into assay mixtures. Following association, 50 µl of a 1:3 slurry of glutathione sepharose beads in association

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buffer was added to each sample, and samples were mixed gently at 4° for 60 min. Beads were washed batch-wise with 5 x 500 µl association buffer and bound protein eluted at 100° for 5 min in 125 µl denaturation buffer (240 mM Tris-HCl, pH 6.8, 20% glycerol, 2% (w/v) SDS, 0.5 M β-mercaptoethanol). Proteins were electrophoresed on
5 12% sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. GST proteins were measured by staining membranes with 0.2% amido black. Membranes were blocked in 5% dry milk (w/v) in Tris-buffered saline, 0.1% Tween-20 (TBS-T) and probed with either a 1:5,000 dilution of anti-Xpress antisera (Invitrogen®) or a 1:2,000 dilution of anti-Ste4 antisera for 2 hr at 25°. Blots
10 were washed extensively with TBS-T and probed with a 1:10,000 dilution of horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit antiserum (Amersham®) for 1 hr at 25°. After washing with TBS-T, blots were developed using the chemiluminescent substrate from Pierce® according to the manufacturers protocol.

GTPαS Binding Assays - A 6 pmol amount of purified His₆-Gαi2 or
15 myristoylated Gαi1 was incubated for 30 min at 25° either alone or with 170 pmol GST or GST-AGS1 in the presence of 1.4 nmol GDP in a total volume of 280 µl assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.6 mM ethylenediaminetetraacetic acid, 5 mM MgCl₂, 0.01% Thesit). A 28 pmol amount of GTPγ³⁵S (1.3 x 10⁶ cpm/pmol) in 70 µl assay buffer was then added and 50 µl aliquots removed at the indicated times for
20 filtration onto nitrocellulose membranes. Filters were washed with 2 x 2 ml ice-cold 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂, air-dried, and bound counts determined in the presence of scintillation fluid.

GTPase Activity Assays - Purified GST and GST-AGS1 or GST-Cdc42 (yeast) at 500 nM were incubated in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM
25 DTT, 0.01% Thesit with 5 µM γ[³²P]-GTP at 25°. At the indicated times, 50 µl aliquots were removed into 750 µl of an ice-cold suspension of 5% charcoal (Norit®) in 50 mM NaH₂PO₄. Samples were vortexed, centrifuged, and 400 µl aliquots of supernatants removed for scintillation counting.

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Guanine Nucleotide Binding Assays - Cultures of CY1316 (Δ gpa1) and CY4600 (Δ gpa1 Δ ste4) carrying pYEX4T1, pYEX4T1-AGS1 or pYEX4T1-CDC42 were grown in low phosphate medium (10 μ M), and GST fusion proteins induced by the addition of 200 μ M CuSO₄. 30 min after copper addition, acid-free [³²P]-PO₄ was also added (50 μ Ci/ml). Cells were grown an additional 5 hr, harvested, washed with cold water, and pellets quick frozen on dry ice. Thawed pellets were lysed by vortexing with beads in 50 mM Tris, pH 7.4, 20 mM MgCl₂, 50 mM NaCl, 0.5% Triton X-100, 1 mM DTT, protease and phosphatase inhibitors. Samples were centrifuged 10 min at 10000 rpm and supernatants bound to 150 μ l glutathione sepharose for 2 hr at 4°. Columns were washed extensively in 50 mM Tris, pH 7.4, 20 mM MgCl₂, 50 mM NaCl, 0.1% Triton X-100 and bound material eluted by heating to 70° in 50 mM Tris, pH 8.8, 20 mM reduced glutathione, 150 mM NaCl, 2% SDS, 20 mM EDTA, 2 mM GDP, 2 mM GTP. Eluted GST fusion proteins were quantitated by SDS-PAGE followed by staining gels in Coomassie blue, and equivalent amounts of GST fusion proteins were spotted onto PEI-cellulose plates. After allowing samples to dry, plates were washed extensively with ddH₂O followed by methanol, dried and developed in 1 M KPO₄, pH 3.4. Dried plates were exposed to X-ray film for analysis.

Abbreviations used herein are as follows: GPCR, G-protein coupled receptor; PAK, p-21 activated protein kinase; MAP kinase, mitogen activated protein kinase; RGS, regulator of G protein signaling; PCR, polymerase chain reaction; IGP, imidazoleglycerolphosphate; AT, 2-aminotriazole; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; GST, glutathione-S-transferase; TBS-T, Tris-buffered saline containing 0.1% Tween-20; DTT, dithiothreitol; GTP α S, guanosine-5'- α -(3-thiotriphosphate); CPRG, chlorophenolred- β -D-galactopyranoside; and IPTG, isopropyl β -D-thiogalactopyranoside.

EXAMPLE 1: Yeast Host Strain for cDNA Library Screening

Upon activation of the pheromone response pathway, *Saccharomyces cerevisiae* normally undergoes Far1-mediated growth arrest (Chang and Herskowitz (1990) *Cell* 63:999-1011; Peter and Herskowitz (1994) *Science* 265:1228-1231). Deletion of the

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FAR1 gene uncouples growth arrest from pheromone-induced transcriptional activation (Chang and Herskowitz (1990) *Cell* 63:999-1011). By fusing promoter sequences from the pheromone-inducible gene *FUS1* to the coding sequences of *HIS3*, expression of IGP dehydratase (encoded by the *HIS3* gene) can be regulated by activation of the

5 pheromone response pathway (Price et al. (1995) *Mol. Cell. Biol.* 15:6188-6195). .

Indeed, this regulated construct has proven useful in the identification of yeast mutants with constitutive pheromone pathway activation in the absence of a functional heterotrimer (Stevenson et al. (1992) *Genes Dev.* 6:1293-1304; Stevenson et al. (1995) *Genes Dev.* 9:2949-2963). Therefore, a *his3* strain carrying the *fus1-HIS3* construct can

10 be made conditional for growth upon pheromone pathway activation by culturing this strain in medium lacking histidine. In a similar fashion, *fus1* promoter sequences can be fused to β -galactosidase gene (*lacZ*) sequences, and pheromone pathway activation quantitated by colorimetric assays. In practice, the *fus1* promoter activity of these constructs is often not entirely repressed in the absence of pheromone pathway

15 activation (King et al. (1990) *Science* 250:121-123). To counteract this in strains carrying the *fus1-HIS3* reporter, low levels of AT can be added to the culture medium to fully repress *HIS3* activity (Manfredi et al. (1996) *Mol. Cell. Biol.* 16:4700-4709).

In searching for non-receptor modulators of the pheromone response pathway that act independently of GPCRs, a yeast strain lacking its native GPCR, Ste3, and its

20 native heterotrimeric $G\alpha$ subunit, Gpa1 (CY1316; see Table 1) was utilized. We introduced into this strain plasmid CP1183, encoding a chimeric $G\alpha$ construct containing the first 41 amino acids of Gpa1 fused to amino acids 36-356 of human $G\alpha i2$. This chimeric construct is capable of functionally coupling to the yeast $G\beta\gamma$ dimer, allowing for full growth repression in the presence of 1 mM AT.

25

EXAMPLE 2: Screening of a Human Liver cDNA Library for Pheromone Response Pathway Activators

In validating the pheromone pathway activator screen a cDNA library derived from adult human liver was chosen. The human liver library was chosen as a potential

30 general source of regulators. This cDNA library was ligated into the high-copy 2 μ -based

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yeast vector pYES2. Sequences were ligated downstream of the yeast *GALI* promoter, making expression of cDNAs conditional upon the addition of galactose to the growth medium. The combination of a high-copy number vector and a strong galactose-inducible promoter increases the ability to detect even weak activators of the pheromone response pathway. A schematic of the pathway and yeast screen is shown respectively in Figs. 1A, 1B, and 2.

Yeast strain CY1316/1183 was transformed with each of the libraries and transformation mixtures plated on a series of agar plates containing sucrose to select for primary transformants. Sucrose was used as a carbon source rather than glucose, as glucose actively represses the *GALI* promoter (Schneider et al. (1991) *Meth. Enz.* 194:373-388). After allowing growth of primary transformants at 30° for 22 hr, replica plates were made onto galactose medium lacking histidine and containing 1 mM AT. Colonies appeared after approximately 2-3 d when grown at 30°. Those transformants unable to produce *HIS3* still must deplete their intracellular stores of histidine prior to the onset of cell stasis, leading to background growth on the replica plates. By replica plating transformants before visible colonies have formed background growth is kept minimal, allowing for clearer selection of colonies that are true histidine prototrophs. If necessary, a second replica plating to identical selective medium can be performed to distinguish true histidine prototrophs from those colonies exhibiting delayed cell inviability.

Growing colonies were recovered onto fresh sucrose plates containing histidine, and tested in a secondary screen on both glucose and galactose plates lacking histidine and containing 1 mM AT to identify galactose-dependent histidine prototrophs. Many of the colonies growing on the initial selection plates did not show galactose-dependent growth in this subsequent screen. These colonies may arise from several sources, including spontaneous AT resistance, genomic alterations to activate the pheromone response pathway, and spontaneous mutations leading to reversion of the genomic *his3* allele or constitutive activation of the *FUS1* promoter. By expressing cDNAs under control of an inducible promoter, most colonies with plasmid-independent growth were eliminated by this rapid and simple secondary screen (see Table 2).

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Plasmids were isolated from strains showing galactose-dependent growth and used to re-transform naïve CY1316/1183 strains. Transformants were again plated onto sucrose medium, then replica-plated onto both galactose and glucose medium lacking histidine and containing 1 mM AT. Those plasmids that conferred universal galactose-
5 dependent growth on this medium were considered to carry cDNAs encoding pheromone pathway activators. Eight such plasmids were identified from these screens, each containing the same open reading frame downstream of the *GALI* promoter (designated L1, see Table 2).

Table 2. Characteristics of yeast screens.

A. Activator Screens				
<u>Library</u>	<u>Transformants</u>	<u>Primary screen</u>	<u>Secondary screen</u>	<u>Retransformation</u> <u>ORF(Frequency)</u>
Liver ~3 x 10 ⁵	146	16	8	L1 (8)

5

CY1316/1183 transformants were screened for colony growth on galactose medium lacking histidine and containing aminotriazole (Primary screen), then for galactose-dependent (Secondary screen) and plasmid-dependent (Retransformation) colony growth. Open reading frames (ORF) downstream of the yeast *GAL1* promoter are designated L1 with their frequency of occurrence in parenthesis. See text for details.

10

B. Inhibitor Screen

<u>Library</u>	<u>Transformants</u>	<u>Primary screen</u>	<u>Secondary screen</u>	<u>Retransformation</u>
Liver ~1.3 x 10 ⁶	238	1	1	

CY1141/1451-AGS1/2440 transformants were screened for colony growth on galactose medium lacking arginine and containing canavanine (Primary screen), then for galactose-dependent (Secondary screen) and plasmid-dependent (Retransformation) colony growth. The sole isolate from this screen carried an ORF downstream of the *GAL1* promoter encoding human RGS5.

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All were found to contain the same open reading frame of 846 bases encoding a protein of 281 amino acids, shown in Figures 3A and 3B (the nucleotide sequence of which is also shown in SEQ ID NO: 1 and the encoded amino acid sequence of which is also shown in SEQ ID NO: 2). The open reading frame of these isolates was designated
5 AGS, for Activator of G-protein Signaling. Each isolate contained varying amounts of 5' and 3' untranslated sequence, and therefore appears to have been selected independently. A composite AGS nucleotide sequence containing the longest identified 5' and 3' untranslated regions of AGS is shown in SEQ ID NO: 3.

10 **EXAMPLE 3: AGS-Mediated Activation of the Pheromone Pathway Occurs at the Level of the Heterotrimeric G-Protein**

Strains carrying pYES2-AGS1 exhibit galactose-dependent growth as well as galactose-dependent reporter gene activity (in strains also carrying the *FUS1p-lacZ* construct). The galactose-dependent growth phenotype conferred upon strain
15 CY1316/CP1183 by pYES2-AGS was further characterized by genetic analysis. pYES2-AGS fails to confer galactose-dependent growth on medium lacking histidine and containing 1 mM AT to strains CY4600, CY12444 and CY12970, containing, respectively genomic disruptions of *STE4*, *STE5*, and *STE20* (see Table 1). This lack of growth is consistent with AGS activation of signaling occurring through the pheromone
20 response pathway ($G\beta\gamma \rightarrow \text{PAK} \rightarrow \text{MAPK}$ cascade). Neither were the isolates of strains carrying an episomal copy of human RGS4 under control of the constitutive *PGK1* promoter able to confer galactose-dependent growth. Furthermore, the inability of AGS to confer growth on a strain deleted for *Gβ* is consistent with AGS-mediated activation occurring at or above the level of the heterotrimeric G-protein.

25 Plasmid pYES2-AGS was also unable to confer galactose-dependent growth on medium lacking histidine and containing 1 mM AT to strain CY1316/CP5533, carrying the G204A allele of *GPAL(1-41)-Goi2*. Three independent isolates each of strains CY1316/pYES2 and CY1316/pYES2-AGS carrying plasmid CP5533 (G204A), and one isolate each of strains CY1316/pYES2 and CY1316/pYES2-AGS carrying plasmid
30 CP1183 (WT) were grown on sucrose medium for 2 d. Equal numbers of cells were

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then resuspended in sterile H₂O and spotted onto galactose medium lacking histidine and containing 1mM AT. Cells were grown 2 d at 30° prior to photography to visualize growth. Neither the isolates of strains CY1316/pYES2 or CY1316/pYES2-AGS carrying plasmid CP5533 (G204A), exhibited growth above that seen with control strain
5 CY1316/pYES2 carrying plasmid CP1183 (WT). By contrast, positive control strain CY1316/pYES2-AGS carrying plasmid CP1183 (WT) exhibited significant colony growth. The G204A mutation is analogous to the G226A mutation in Gas which renders this protein unable to form a high affinity Mg²⁺•GTP complex (Lee et al. (1992) *J. Biol. Chem.* 267:1212-1218). The G204A mutation in the chimeric Gpa1(1-
10 41)-Gai2 also appears to render it incapable of activation, as evidenced by its failure to transduce a receptor-mediated signal to activate *fus1p-HIS3* transcription.

To rule out that AGS expression was affecting the expression of the Gpa1(1-41)-Gai2 chimera, membrane preparations from strains CY1316/CP1183 and CY1316/CP5533 expressing either the pYES2 vector or the pYES2-AGS plasmid and
15 grown for 20 hr in galactose-containing medium were analyzed by immunoblot analysis using antisera specific for the C-terminus of Gai2. No significant differences in chimeric Gpa1(1-41)-Gai2 expression were detected in any of these strains.

These data strongly suggest that AGS activation of the pheromone response pathway is occurring at the level of the heterotrimeric G protein. Furthermore, the
20 inability of AGS to activate the pheromone response pathway in the presence of a Gα that is defective in GDP-GTP exchange, and the opposing effects of AGS function and RGS4 function on pheromone pathway activation strongly suggest that AGS functions by facilitating GTP exchange on the heterotrimeric Gα.

Finally, the Gα selectivity profile of AGS1 was determined. The pYES/*FUS1p-lacZ* transformants in strain CY1316 (no Gα), or CY1316 derivatives carrying an
25 integrated copy of a *GPA1*-human Gai2 fusion construct (CY1141), a *GPA1*-human Gα i3 fusion construct (CY7967), a rat Gas construct (CY8342), a *GPA1*-human Gα16 fusion construct (CY9603), or yeast *GPA1* (CY9571) (all under the control of the yeast *GPA1* promoter) were grown in selective liquid medium containing either glucose or

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galactose. Relative β -galactosidase activity was then determined on aliquots of permeabilized cells (Table 3).

TABLE 3

5

 β -galactosidase activity

Strain	VECTOR		AGS1	
	Glucose	Galactose	Glucose	Galactose
10 No $G\alpha$	103.4 +/- 5.7	76.0 +/- 6.9	145.0 +/- 5.9	140.1 +/- 4.4
$G\alpha i2$	11.1 +/- 0.9	8.7 +/- 0.5	5.6 +/- 0.6	124.8 +/- 4.1
15 $G\alpha i3$	10.0 +/- 0.6	9.8 +/- 0.6	5.6 +/- 0.7	63.3 +/- 7.8
$G\alpha s$	13.3 +/- 1.0	11.9 +/- 0.8	10.1 +/- 0.5	6.4 +/- 1.1
20 $G\alpha 16$	23.6 +/- 0.7	24.3 +/- 1.6	22.5 +/- 1.2	22.5 +/- 1.6
$G\alpha 1$	5.6 +/- 0.3	5.4 +/- 0.3	3.6 +/- 0.5	7.6 +/- 0.8

Analysis of the $G\alpha$ selectivity profile for AGS1 in yeast strains carrying a *fus1p-lacZ* reporter construct and expressing different mammalian $G\alpha$ constructs indicated $G\alpha i$ specificity. Expression of AGS1 resulted in high levels of β -galactosidase activity in strains expressing $GPAL_{(1-41)}-G\alpha i2$ or $GPAL_{(1-41)}-G\alpha i3$, but not in strains expressing $G\alpha s$, $GPAL_{(1-41)}-G\alpha 16$ or $GPAL$ itself (Table 3).

Immunoblot analysis using polyclonal serum against $G\alpha 1$ and $G\alpha s$ indicated
 30 that each of the host strains expressed equivalent amounts of $G\alpha$ and that expression of

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AGS1 had no significant effect on the steady-state levels of any of these G α proteins. Similar experiments indicated that expression of AGS1 had no significant effect on the steady-state levels of yeast G β .

5 **EXAMPLE 4: A Counter-Screen for Pheromone Pathway Inhibitors**

With the identification of AGS1 as a receptor-independent mammalian activator of the yeast pheromone response pathway, a novel screen was devised to identify pheromone pathway inhibitors. For this screen, a strain was created that had its pheromone pathway constitutively activated by AGS1. Then, a human liver cDNA
10 library in vector pYES2 was introduced into this strain in an attempt to identify cDNAs that, when expressed, counteracted AGS1 function. This screen, therefore, can identify proteins that both directly and indirectly regulate AGS1 activity.

In this screen, the marker gene *CAN1* was employed which encodes a yeast arginine permease that can transport the arginine analog canavanine (Sikorski (1991)
15 *Meth. Enz.* 194:302-318). Accordingly, cells expressing Can1 and cultured in the absence of arginine can transport canavanine and incorporate it into nascent proteins, leading to cell death. By fusing *CAN1* coding sequences downstream of the *FUS1* promoter and introducing it into yeast strain CY1141 (Table 1), this strain can be made conditionally non-viable upon pheromone pathway activation. Into this strain the AGS1
20 coding sequences fused downstream of the constitutive yeast promoter *PGK1* were introduced (Schena et al. (1991), *Meth. Enz.* 194:389-398). Expression of AGS1 in this strain led to constitutive pheromone pathway activation, as evidenced by growth on medium lacking histidine and inhibition of growth on medium lacking arginine and containing canavanine.

25 The observation was made that the constitutive expression of human RGS4 interfered with AGS1 function. Thus, RGS4 was ligated downstream of the *GALI* promoter in pYES2 and used as an initial proof-of-concept test for the inhibitor screen. When introduced into CY1141 carrying *PGK1p*-AGS1 and *FUS1p*-*CAN1* expression constructs, pYES2-RGS4 conferred galactose-dependent growth on medium lacking
30 arginine and containing canavanine. The amount of canavanine used in this test (200

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µg/ml medium) was determined to be optimal in suppressing the growth of pYES2 vector transformants without causing general lethality. Therefore the strain was transformed with the human liver cDNA library and a inhibitor screen analogous to the activator screen was carried out. A schematic of the inhibitor screen is shown in Figure 2 and the screen characteristics are shown in Table 2. A higher level of plasmid-independent colony formation was expected from this screen relative to that of the activator screen, as any genomic mutation that abolishes pheromone pathway signalling should confer growth under restrictive conditions. This background, again, was easily eliminated with a secondary screen comparing growth on glucose medium versus growth on galactose medium. In addition, a higher level of general background growth in this screen was encountered compared with the activator screen. An additional replicating step in the secondary screen eliminated much of this background (see Table 3). In the initial screen of 1.3×10^6 primary transformants, a single cDNA clone (L15) was isolated that was capable of conferring growth in a galactose-specific manner under selective conditions. Sequencing of the 1.7 kilobase cDNA insert (SEQ ID NO: 24) revealed it to encode human RGS5 (SEQ ID NO: 25). Human RGS5 is an ortholog of mouse RGS5, which has been shown to bind *in vitro* to both G α i and G α o (Chen et al. (1997) *J. Biol. Chem.* 272:8679-8685). The ability to isolate one member of a family of known down-regulators of heterotrimeric G-protein signaling pathways not only serves to validate this inhibitor screen, but also to reinforce the mechanism of action of AGS1.

In summary, the functional redundancy found in many key regulatory pathways of both yeast and mammalian cells allows for the development of screens designed to identify specific modulators of these pathways. The ability to isolate both mammalian activators and inhibitors of the yeast pheromone response pathway demonstrates the utility of these yeast-based functional screens. Moreover, the short time frame required for each screen (1-2 weeks), as well as the relatively high throughput (Table 3), allows for rapid saturation screens for a cDNA library of interest. Accordingly, the implementation of these screens using cDNA libraries generated from different normal or disease-specific tissue sources can be used to identify other novel pathway activators or inhibitors.

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EXAMPLE 5: Characterization of Isolated cDNA Clone

The open reading frame of the 8 isolated clones encodes a 281 amino acid protein with homology to small ras-like G proteins. This newly cloned protein was termed AGS. A search of GenBank revealed that AGS exhibits homology (96% at the amino acid level, 85% at the nucleotide level) with a previously identified glucocorticoid-induced ras-related protein in mouse (accession number AF009246). Sequence comparison with the yeast genome indicated that AGS shares the highest degree of homology with *RSR1/BUD1* (45% amino acid identity within amino acids 25-125 of AGS) and both *RAS1* and *RAS2* (43% amino acid identity within amino acids 25-125 of AGS). An alignment of AGS with representatives of all major classes of small G proteins in humans (Figures 4A and 4B) indicates that AGS is likely to be the founding member of a novel class of small G proteins in humans.

Though AGS is clearly a member of the ras superfamily, it has several unique structural features, including both N- and C-terminal extensions not seen in most small G proteins. AGS also has alterations in several amino acids that are normally highly conserved in all small G proteins. Indeed, AGS shares alterations at three key amino acid residues with the recently identified G proteins Rnd1, Rnd2 and RhoE/Rnd3 (Foster et al. (1996) *Mol. Cell. Biol.* 16:2689-2699; Nobes et al. (1998) *J. Cell Biol.* 141:187-197) (see Figure 5). RhoE was shown to be deficient in GTP hydrolysis activity, and this deficiency could be restored by alteration of these three amino acids to their highly conserved counterparts (Foster, R. et al. (1996) *Mol. Cell. Biol.* 16:2689-2699). Though AGS shares these three amino acid alterations with Rnd1, Rnd2 and RhoE/Rnd3, it does not share the consensus rho effector (amino acids 42-50 in Rnd1) or rho insert (amino acids 132-140 in Rnd1) domains of these proteins. AGS, therefore, does not appear to be a member of the rho family of ras-like proteins.

EXAMPLE 6: Mutational Analysis of AGS Protein

A series of point mutations were created in AGS to analyze their effects on pheromone pathway activation, as measured by galactose-dependent growth in the absence of histidine. Mutations at conserved glycine residues 31 and 36, which are predicted to be in the P site, render AGS unable to confer histidine prototrophy to cells.

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Surprisingly, mutation of another conserved glycine in the G' site, glycine-81, does not appear to affect the function of AGS, even with growth on medium containing 20 mM AT. This glycine mutation is analogous to the G(226)A mutation of G α s and the G204A mutation of G α i2, and would normally be predicted to by an inactivating mutation.

5 A number of other mutants (listed below in table 4) were tested in the vector pYES2 in CY1141 on medium lacking histidine and containing 1 mM aminotriazole as described above. The β -galactosidase activities (relative to wild-type AGS1) of all of the mutants tested are as follows.

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Table 4

<u>Construct</u>	<u>β-gal activity (glucose)</u>	<u>β-gal activity (galactose)</u>
Wild type	8.5	100
G(31)V	11.1	8.2
5 G(36)V	10.3	10.9
G(81)A	9.3	69.6
S(33)G	8.4	68.6
S(80)A	10.1	104.0
N(82)G	12.2	66.0
10 F(44)N	not tested	not tested
S(33)G; S(80)A	9.3	39.1
S(33)G; N(82)A	10.1	37.2
S(80)A; N(82)G	9.9	92.6
S(33)G; S(83)A; N(82)G	7.7	71.1
15 S(33)G; S(80)A; N(82)G;		
G(81)A	12.0	125.5
K(225)E; K(226)E	13.0	81.0
C(278)S	14.4	16.4
Vector	10.1	8.9

20

The G(31)V mutant carried a glycine to valine alteration at residue 31 (G31V), an absolutely conserved residue in the P-loop of monomeric G proteins that makes critical contacts with the α - and β -phosphates of both GDP and GTP (Valencia et al. (1991) *Biochemistry* 30:4637-4648). The C(278)S mutant carried a cysteine to serine alteration at residue 278 (C278S) within the C-terminal CAAX box. This cysteine is a major site of lipid modification for most members of the ras superfamily. Neither of these mutations appeared to destabilize the AGS1 protein, as immunoblot analysis of both N-terminally His₆- and GST-tagged mutant proteins showed no significant change in the steady-state levels relative to wild-type AGS1. The introduction of either of these two amino acid changes in AGS1, however, severely impaired its ability to activate the

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pheromone pathway consistent with AGS1 function requiring both guanine nucleotide binding and post-translational lipid modification.

EXAMPLE 7: Effect of STE14 on AGS Function

5 The yeast strain used in the isolation of AGS contained a genomic disruption of the *STE14* locus, which encodes a farnesyl cysteine:carboxyl methyltransferase known to carboxymethylate the yeast G α , Ste18, Ras1 and Ras2 proteins as well as a-factor (Hrycyna and Clarke (1990) *Mol. Cell. Biol.* 10:5071-5076; Marr et al. (1990) *J. Biol. Chem.* 265:20057-20060; Hrycyna et al. (1991) *EMBO J.* 10:1699-1709). Disruption of
10 *ste14* has been found to reduce background signaling through the pheromone response pathway in strains carrying chimeric G α constructs. Because AGS appears to encode a small ras-related G protein with a C-terminal CaaX box sequence consistent with farnesylation, and because small G proteins are often carboxymethylated after farnesylation, we examined the effect of addition of an episomal copy of *STE14* on AGS
15 function. *STE14* gene expression appeared to enhance the *fus1-HIS3* mediated growth of strains expressing AGS, though an increase in background growth on glucose medium was also evident.

EXAMPLE 8: Tissue Expression of AGS mRNA

20 The tissue-specific expression of AGS in mRNAs isolated from various human tissues was measured. A ³²P-labeled DNA probe was made from the full length coding sequence of AGS and hybridized under stringent conditions to mRNA Northern blots and an mRNA dot blot generated from human tissues and human cancer cell lines. The AGS probe hybridized to a major transcript at approximately 2 kb and a minor transcript
25 at approximately 5 kb. The major transcript hybridized most strongly with mRNAs derived from liver, skeletal muscle, heart, kidney, brain and placenta, prostate and bone marrow tissues, as well as from a HeLa cell line. Interestingly, the relative ratio of the major and minor transcripts varies with tissue source, and the major transcript is almost entirely absent from mRNA derived from fetal liver. Upon longer exposure times,
30 hybridization to the AGS probe is detectable in all mRNA samples, probably indicating low-level of AGS expression in all tissues.

EXAMPLE 9: Chromosomal localization of AGS1

In a further characterization of the AGS 1 gene, the chromosomal locus of AGS1 was determined according to the protocol of Genome Systems, Inc. (St. Louis, MO). A full length AGS1 cDNA (SEQ ID NO: 1) was used to identify a P1 genomic clone from a human library. This P1 clone, with fluorescence in situ (FISH), localized the AGS1 gene to chromosome 17 in band 17p11.2. AGS1 sequences were localized to 38% of the distance from the centromere to the telomere of chromosome arm 17p, and 71 out of 80 metaphase chromosome spreads that were analyzed exhibited specific labeling with an AGS1 probe. The homolog of AGS1 has been (as described in Example 15) localized to chromosome 22 in band 22q13.1. A P1 genomic clone carrying AGS1 has been analyzed by PCR amplification using the oligo sequences 5'TTCTCGCGGATGTACATGA33' (SEQ ID NO: 43) and 5'TCCACCGCAAGTTCTACTCC3' (SEQ ID NO: 44) and verified, by size analysis, to contain an AGS1 DNA sequence.

EXAMPLE 10: Expression of AGS Protein in Host Cells

Coding sequences for AGS were amplified by PCR for expression in yeast, *E. coli*, insect, and mammalian cells. These sequences were ligated downstream of coding sequences for either GST or His6 epitopes, and protein expression levels were monitored by immunoblot analysis using antisera directed against the respective fusion partners. Inducible expression of GST-tagged AGS in yeast and bacteria was easily detectable. The expression of mutant forms of AGS in yeast, in which glycine residues 36 and 81 were changed, respectively, to valine and alanine was also detectable, as was the expression of His6-tagged AGS in insect cells. A His6-tagged version of AGS was also introduced into the mammalian cell line HEK293, and stable transfectants generated. The expression of tagged AGS mRNA in these cell lines was detectable, but expression of AGS protein was not, indicating that AGS protein may be unstable in this cell line.

However, both wild type and AGS1 mutant G(31)V have been expressed and detected in mammalian CHO-K1 and COS-7 cells and both polypeptides are membrane associated. No AGS1 protein is detectable in the soluble fraction. In addition, in yeast,

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expression of wild type, G(31)V, G(36)V, G(81)A, and S(33)G; S(80)A; N(82)G AGS1 fusions to GST have been detected in whole cell yeast extracts. An *in vitro* translation reaction was performed to verify expression of His₆-tagged AGS from pcDNA3.1-HisC-AGS, as well as to verify expression of non-tagged AGS from the original pYES2-AGS isolate. Expression of ³⁵S-labeled AGS was readily detectable from these plasmids.

GST- and His₆-fusions of AGS were purified by affinity chromatography from *E. coli* or yeast (*S. cerevisiae*) and insect cells using, respectively, glutathione sepharose and Ni-agarose matrices. A GST-fusion of Cdc42 was also purified from *E. coli* and yeast. Purification was monitored by SDS-polyacrylamide gel electrophoresis and immunoblotting. Large amounts of purified soluble GST-fusion proteins can be readily obtained from bacterial cultures and yeast cultures. GST-AGS appears to be relatively unstable during purification, even with buffers containing high levels of Mg²⁺ and either GDP or GTP.

EXAMPLE 11: Association of Heterotrimeric G-Protein Subunits with GST-AGS1

Purified GST or GST-AGS1, from yeast was incubated with crude extract from Sf9 cells expressing human His₆-Gαi2 or yeast Gβ-His₆Gγ. Gαi2 extracts were pre-incubated with excess GDP or GTPγS prior to incubation with immobilized proteins. After extensive washing, bound Gαi2 and Gγ were detected with antiserum specific to the hexahistidine tag, and bound Gβ was detected with antiserum specific to Ste4. This GST-AGS1 construct expressed in yeast is functional, conferring histidine prototrophy to CY1316/1183.

EXAMPLE 12: AGS1 Stimulates GTP Binding to Gα Subunits

GST-AGS1 was assayed for its ability to enhance binding of GTPγS to purified Gαi1 and Gαi2. Under conditions used to monitor G-protein activation by a GPCR (Sato et al. (1996), *J. Biol. Chem.* 271:30052-30060), addition of AGS1 clearly enhanced GTPγS binding on both Gα proteins (approximately 25% purified Gα was bound to GTPγS at 40 min in the presence of AGS1 as compared to approximately 7%

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bound at 40 min in the absence of AGS1). AGS1 also was able to enhance GTP γ S binding to a similar extent on purified brain heterotrimeric G-protein, where the majority of G α in the heterotrimer is G α_o , suggesting AGS1 also functions on G α_o and functions independently of the presence of G $\beta\gamma$. These observations, in combination with the *in vivo* epistasis and G α selectivity data, indicate that AGS1 likely functions by catalyzing guanine nucleotide exchange on selected G α proteins. Purified GST-AGS1 alone does not bind GTP γ S under these conditions, nor does it bind GTP γ S when presented in the absence of excess GDP. Two control experiments were done to assess where the labelled GTP γ S was being bound in these assays. First, Ni-NTA agarose and glutathione sepharose were used to pull out either His₆-Gai2 or GST-AGS1 after 40 min, and the majority of GTP γ S counts were found on the Ni-NTA column. Second, purified Gai2 was pre-loaded with non-labelled GTP γ S, dialyzed, and used in the assay. This led to an approximately 70% decrease in label bound. The converse experiment, in which purified GST-AGS1 was pre-loaded with cold GTP γ S prior to running the assay showed only an approximately 15% decrease in label bound.

EXAMPLE 13: Induced Expression of AGS-1 Activities ERK1/ERK2 Pathway

AGS1 and a His₆-tagged version of AGS1 have also been constructed in the ecdysone (ponasterone)-inducible mammalian expression vector pIND (Invitrogen). using standard techniques. Upon induction, assays for determining activation of MAP kinase pathways were performed on cells carrying inducible pIND-AGS1 constructs. These assays indicated that in HEK293 cells, the ERK1/ERK2 pathway is activated upon induction of expression of AGS1 and that this activation is abolished by pretreatment of cells with pertussis toxin (Ptx) (Table 5).

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Table 5

<u>Sample</u>	<u>ERK1/ERK2 activation (cpm) +/- S.E.M.</u>
5 pIND-AGS1 (uninduced)	21710 +/- 1530
pIND-AGS1 (induced)	32070 +/- 1270
10 pIND-AGS1 + Ptx (uninduced)	23700 +/- 960
pIND-AGS1 + Ptx 15 (induced)	26460 +/- 400

EXAMPLE 14: GTPase Analysis of AGS1

AGS1 has been determined to have GTPase activity. Using a standard GTPase assay as described herein, it was determined that purified GST-AGS1 preparations have significant GTPase activity. For example, at 120 min, the amount of phosphate released into the buffer was 4.8 pmol per 25 pmol GST (background is 4 pmol), 28 pmol per 25 pmol GST-AGS1, and 33 pmol per 25 pmol GST-Cdc42. Also, there was a time and AGS1 dose-dependence for phosphate release. Neither excess phosphate nor excess ATP could significantly affect the degree of phosphate release for GST-AGS1 or GST-Cdc42, but excess unlabelled GDP and GTP inhibited the rate of phosphate release, indicating that both GST-AGS1 and GST-Cdc42 are specifically hydrolyzing GTP (e.g. there is not a contaminating non-specific nucleotidase or phosphatase activity in the preparations).

In addition, it was determined by in vivo labeling with $^{32}\text{PO}_4$ as described herein that AGS1 bound nucleotide much less well than Cdc42. GST-Cdc42, as expected, was mostly bound to GTP after purification from strain CY1316 (pathway on), and GDP

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after purification from strain CY4600 (pathway off). AGS1, however, seemed to be bound only to GTP when purified from either strain background—no GDP was detected at all in either sample. Relative to Cdc42, it is estimated that less than 1% of AGS1 is bound to nucleotide after purification.

5 Taken together, the above biochemistry suggests that AGS1 can only bind guanine nucleotides poorly and has lower affinity for GDP as compared to GTP. Given that its GTPase activity is comparable (mol:mol) with Cdc42, this would suggest that purified AGS1 may have a higher than normal hydrolysis activity. Accordingly, it is likely that only a small fraction of purified AGS1 is active, accounting for the relatively
10 low stimulation of GTPγS binding seen when used in the activation assay and for its apparent instability.

EXAMPLE 15: Cloning and Analysis of a Human AGS1 Homolog.

By sequence comparison, a related human AGS1 homolog was identified from
15 the GenBank database (Accession No.: CAA18456). Alignment of the AGS1 polypeptide sequence to the AGS1 homolog indicated that these proteins were 62% identical when comparing residues 5-280 of AGS1 (SEQ ID NO: 2) to residues 12-227 of the AGS1 homolog (SEQ ID NO: 41). In addition, the cDNAs encoding these proteins were 82% and 84% identical when comparing AGS1 nucleotide bases 102-438
20 and 510-646 of SEQ ID NO: 1 with, respectively, AGS1 homolog nucleotide bases 123-459 and 531-667 of SEQ ID NO: 40. The overall greatest divergence between these two molecules is found in the C-terminal or 3' region of these molecules.

Given the above sequence similarity between AGS1 and the AGS1 homolog, a functional analysis of these related molecules was conducted.. The cDNA for the AGS1
25 homolog was amplified by PCR from a human liver cDNA library and subcloned into pYES2 vector using engineered 5'BamHI and 3' EcoRI sites (as had been done for AGS1 itself). Expression of this homolog did not confer growth to CY1316/1183, nor did it lead to *FUS1p-lacZ* activity in CY1141 (see Table 6). A common internal MscI site in both AGS1 and the AGS1 homolog was taken advantage of in order to exchange
30 the C-terminal regions between these two sequences. The chimeric expression constructs were called AH (for AGS1 head and Homolog tail) and HA (for Homolog

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head and AGS1 tail), and were ligated into the pYES2 vector. Even though the major divergence between these two sequences is in the "tail" region, both growth assays and lacZ assays indicated that the "head" or N-terminal region of AGS1 is important for activity as measured as a function of relative lacZ activities as shown below (Table 6).

5

Table 6 Functional Analysis of AGS1, AGS1 Homolog, and Chimeric Proteins

<u>Construct</u>	<u>β-gal activity (glucose)</u>	<u>β-gal activity (galactose)</u>
pYES2-AGS1	12.0	100.0
pYES2-Homolog	10.2	12.3
pYES2-AH	11.3	52.5
pYES2-HA	13.9	14.0
pYES2 vector	11.3	12.2

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed:

1. An isolated nucleic acid molecule which encodes an AGS protein,
comprising a nucleotide sequence having at least 86% identity to the nucleotide
5 sequence of SEQ ID NO:1, or the complement thereof.
2. The isolated nucleic acid molecule of claim 1, which comprises a
nucleotide sequence having at least 90% identity to the nucleotide sequence of SEQ ID
NO:1 or SEQ ID NO:3, or the complement of SEQ ID NO: 1 or SEQ ID NO: 3.
10
3. The isolated nucleic acid molecule of claim 1, which comprises a
nucleotide sequence having at least 95% identity to the nucleotide sequence of SEQ ID
NO:1 or SEQ ID NO:3, or the complement of SEQ ID NO: 1 or SEQ ID NO: 3.
- 15 4. The isolated nucleic acid molecule of claim 1, which comprises the
nucleotide sequence of SEQ ID NO:1, or the complement thereof.
5. The isolated nucleic acid molecule of claim 1, which comprises the
nucleotide sequence of SEQ ID NO: 3, or the complement thereof.
20
6. The isolated nucleic acid molecule of claim 1, which encodes a protein
that activates G protein-coupled signal transduction in a G protein-coupled receptor
independent manner.
- 25 7. The isolated nucleic acid molecule of claim 1, which is a human nucleic
acid molecule.
8. An isolated nucleic acid molecule comprising a nucleotide sequence
encoding a protein which comprises an amino acid sequence having at least 97% identity
30 to the amino acid sequence of SEQ ID NO:2.

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9. The isolated nucleic acid molecule of claim 8, wherein the protein comprises an amino acid sequence having at least 98% identity to the amino acid sequence of SEQ ID NO:2.

5 10. The isolated nucleic acid molecule of claim 8, wherein the protein comprises an amino acid sequence having at least 99% identity to the amino acid sequence of SEQ ID NO:2.

11. The isolated nucleic acid molecule of claim 8, which encodes a protein
10 comprising the amino acid sequence of SEQ ID NO:2.

12. The isolated nucleic acid molecule of claim 8, wherein said protein activates G protein-coupled signal transduction in a G protein-coupled receptor independent manner.

15

13. The isolated nucleic acid molecule of claim 8, which is a human nucleic acid molecule.

14. A vector comprising the nucleic acid molecule of claim 1.

20

15. The vector of claim 14, which is a recombinant expression vector.

16. A host cell containing the vector of claim 14.

25 17. A method for producing an AGS protein comprising culturing the host cell of claim 16 in a suitable medium such that AGS protein is produced.

18. The method of claim 17, further comprising isolating an AGS protein from the medium or the host cell.

30

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19. A nonhuman transgenic animal which contains cells carrying a transgene encoding an AGS protein.

20. An isolated AGS protein comprising an amino acid sequence having at
5 least 97% identity to the amino acid sequence of SEQ ID NO:2.

21. The isolated protein of claim 20, comprising an amino acid sequence having at least 98% identity to the amino acid sequence of SEQ ID NO:2.

10 22. The isolated protein of claim 20, comprising an amino acid sequence having at least 99% identity to the amino acid sequence of SEQ ID NO:2.

23. The isolated protein of claim 20, comprising the amino acid sequence of
15 SEQ ID NO:2.

24. A fusion protein comprising at least a portion of the AGS protein of claim 20, operatively linked to a non-AGS polypeptide.

25. An antibody that specifically binds the AGS protein of claim 20.
20

26. The antibody of claim 25, which is monoclonal.

27. The antibody of claim 25, which is labeled with a detectable substance.

28. A pharmaceutical composition comprising the protein of claim 20 and a
25 pharmaceutically acceptable carrier.

29. A pharmaceutical composition comprising the antibody of claim 25 and a
30 pharmaceutically acceptable carrier.

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30. A method for identifying a compound that modulates signal transduction in a cell, comprising:

contacting a cell that expresses an AGS protein with a test compound;

determining the effect of the test compound on the activity of the AGS protein;

5 and

identifying the test compound as a modulator of signal transduction based on the ability of the compound to modulate the activity of the AGS protein in the cell.

31. The method of claim 30, wherein the AGS protein comprises an amino
10 acid sequence having at least 97% identity to SEQ ID NO: 2 and stimulates G protein activity in a receptor-independent manner.

32. The method of claim 30, wherein the AGS protein is human.

15 33. The method of claim 30, wherein the AGS protein comprises the amino acid sequence of SEQ ID NO: 2.

34. The method of claim 30, wherein the cell has been engineered to express the AGS protein by introducing into the cell an expression vector encoding the AGS
20 protein.

35. The method of claim 30, wherein the cell has further been engineered to express a G protein α subunit.

25 36. The method of claim 30, wherein the cell is a yeast cell that has been engineered to express a mammalian or chimeric G protein α subunit and the effect of the test compound on the activity of the AGS protein is determined by monitoring a pheromone response pathway in the yeast cells.

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37. The method of claim 36, wherein the yeast cell has been engineered to express a Gpa1-Gαi2 chimeric G protein α subunit.

38. The method of claim 36, wherein the pheromone response pathway in the yeast cells is monitored by measuring the activity of a pheromone responsive promoter in the yeast cells.

39. The method of claim 30, wherein the effect of the test compound on the activity of the AGS protein is determined by monitoring the ability of the test compound to bind to the AGS protein.

40. The method of claim 30, wherein the effect of the test compound on the activity of the AGS protein is determined by monitoring the ability of the test compound to modulate the interaction of the AGS protein with a target molecule.

41. The method of claim 40, wherein the target molecule is a G protein.

42. A method for modulating G protein coupled signal transduction in a cell comprising contacting a cell with an agent which modulates AGS protein activity or AGS nucleic acid expression such that G protein coupled signal transduction is modulated in the cell, when compared with G protein coupled signal transduction in the cell in the absence of the agent.

43. The method of claim 42, wherein the cell-associated activity is a G-protein mediated activity.

44. The method of claim 42, wherein the agent stimulates an AGS protein activity or AGS gene expression.

45. The method of claim 42, wherein the agent is an active AGS protein.

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46. The method of claim 42, wherein the agent is a nucleic acid encoding an AGS that has been introduced into the cell.

47. The method of claim 42, wherein the agent inhibits an AGS protein
5 activity or AGS gene expression.

48. The method of claim 42, wherein the agent is an antisense AGS nucleic acid molecule.

10 49. The method of claim 42, wherein the agent is an antibody that specifically binds to an AGS protein.

50. The method of claim 42, wherein the cell is present within a subject and the agent is administered to the subject.

15

51. A method for treating a subject having a disorder characterized by an aberrant AGS protein activity or nucleic acid expression comprising administering to the subject an AGS modulator such that treatment of the subject occurs.

20 52. A method for detecting the presence of an AGS protein in a biological sample comprising contacting a biological sample with an agent capable of detecting the AGS protein or mRNA such that the presence of the AGS protein is detected in the biological sample.

25 53. The method of claim 52, wherein the agent is a labeled or labelable nucleic acid probe capable of hybridizing to an AGS mRNA.

54. The method of claim 52, wherein the agent is a labeled or labelable antibody capable of specifically binding to an AGS protein.

30

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55. A method for identifying a compound that activates a signal transduction pathway in a cell, said method comprising,

providing a cell that undergoes a measurable change when the signal transduction pathway is activated;

5 contacting the cell with a test compound; and
determining if the test compound causes a measurable change to
thereby identify the test compound as a modulator of the signal transduction
pathway.

10 56. The method of claim 55, wherein said signal transduction pathway is a G protein signaling pathway.

57. The method of claim 56, wherein said cell is a yeast cell.

15 58. The method of claim 57, wherein said G protein signaling pathway is the yeast pheromone response pathway.

59. The method of claim 58, wherein said compound is a polypeptide encoded by a nucleic acid molecule.

20

60. The method of claim 59, wherein said nucleic acid comprises a nucleotide sequence having at least 70% identity to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

25 61. The method of claim 59, wherein said nucleic acid comprises a nucleotide sequence having at least 80% identity to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

30 62. The method of claim 59, wherein said nucleic acid comprises a nucleotide sequence having at least 90% identity to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

63. The method of claim 59, wherein said nucleic acid comprises a nucleotide sequence having at least 95% identity to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

5

64. The method of claim 59, wherein said nucleic acid comprises the nucleotide sequence of SEQ ID NO:1.

65. The method of claim 59, wherein said nucleic acid comprises the
10 nucleotide sequence of SEQ ID NO: 3.

66. The method of claim 59, wherein said polypeptide activates G protein signaling in a G protein coupled-receptor independent manner.

15 67. The method of claim 59, wherein said nucleic acid comprises a nucleotide sequence which is a human.

68. The method of claim 59, wherein said polypeptide comprises an amino acid sequence having at least 97% identity to the amino acid sequence of SEQ ID NO:2.

20

69. The method of claim 59, wherein the polypeptide comprises an amino acid sequence having at least 98% identity to the amino acid sequence of SEQ ID NO:2.

70. The method of claim 59, wherein the polypeptide comprises an amino
25 acid sequence having at least 99% identity to the amino acid sequence of SEQ ID NO:2.

71. The method of claim 59, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2.

30 72. The method of claim 30, wherein the compound is a nucleic acid encoding a polypeptide capable of inhibiting the activity of the AGS protein.

73. The method of claim 72, wherein said nucleic acid comprises the sequence provided in SEQ ID NO: 24.

5 74. The method of claim 72, wherein said nucleic acid encodes the polypeptide sequence provided in SEQ ID NO: 25.

75. The method of claim 30, wherein the cell further comprises a nucleic acid encoding an inhibitor of the AGS protein.

10

76. The method of claim 75, wherein said nucleic acid comprises the sequence provided in SEQ ID NO: 24.

77. The method of claim 75, wherein said nucleic acid encodes the
15 polypeptide sequence provided in SEQ ID NO: 25.

78. The method of claim 75, wherein said ability of the test compound to modulate the activity of the AGS protein indicates that the test compound is a modulator of the inhibitor of the AGS protein.

20

FIG. 1B

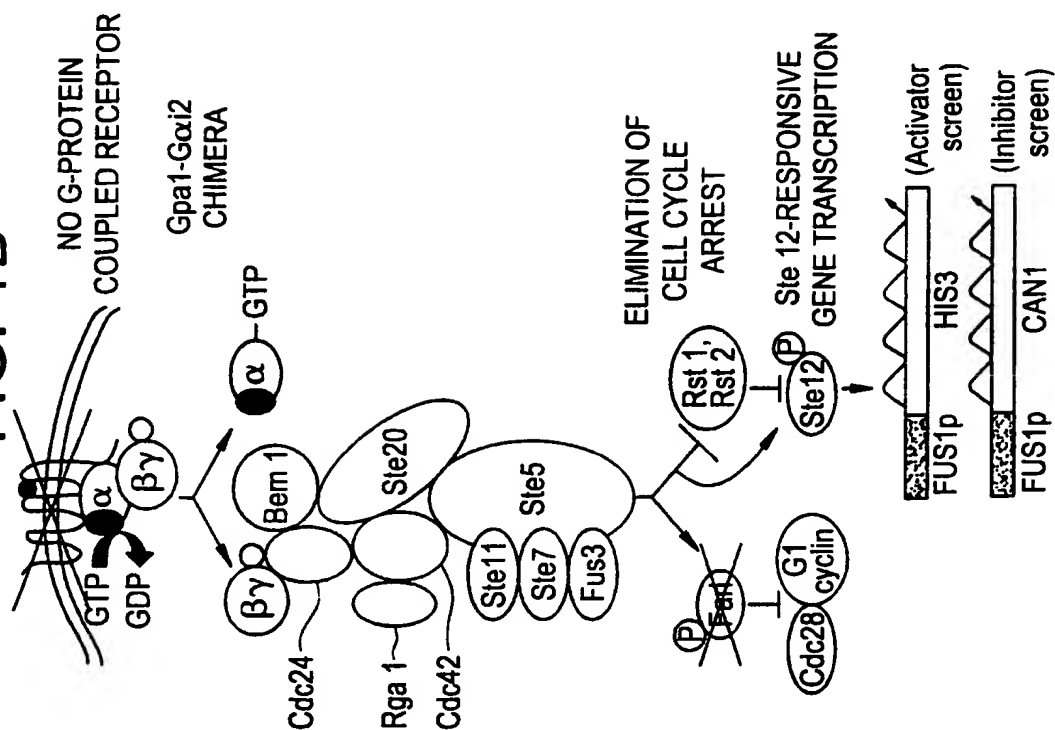
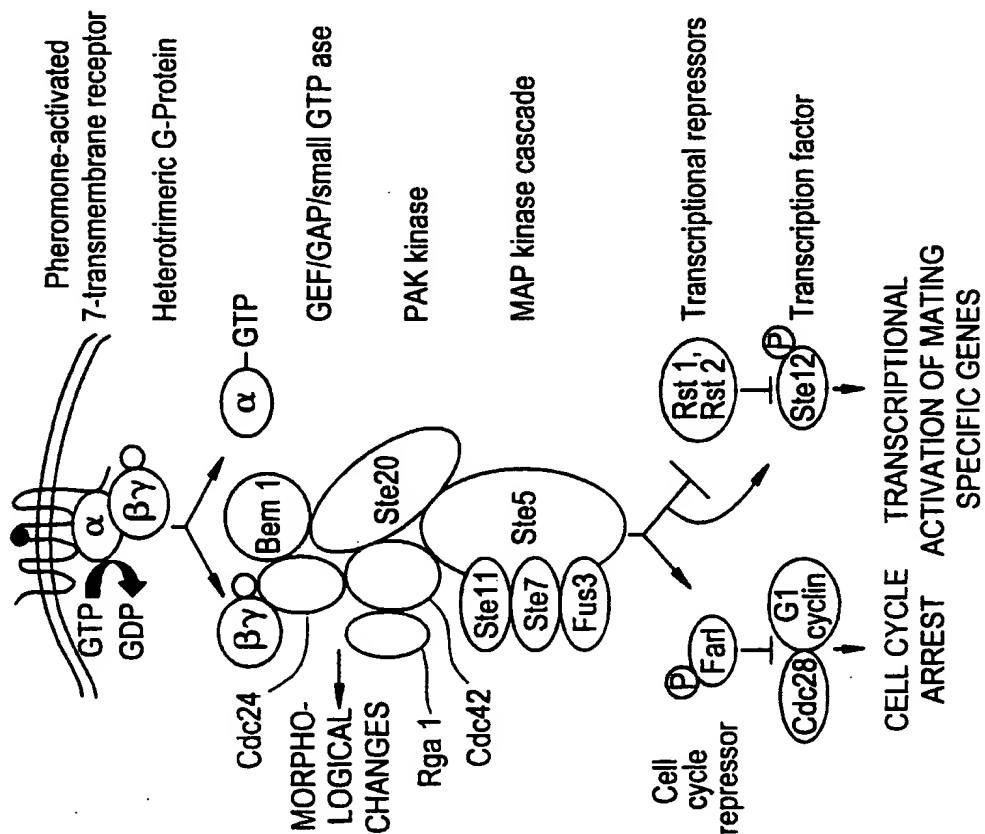


FIG. 1A



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FIG. 2

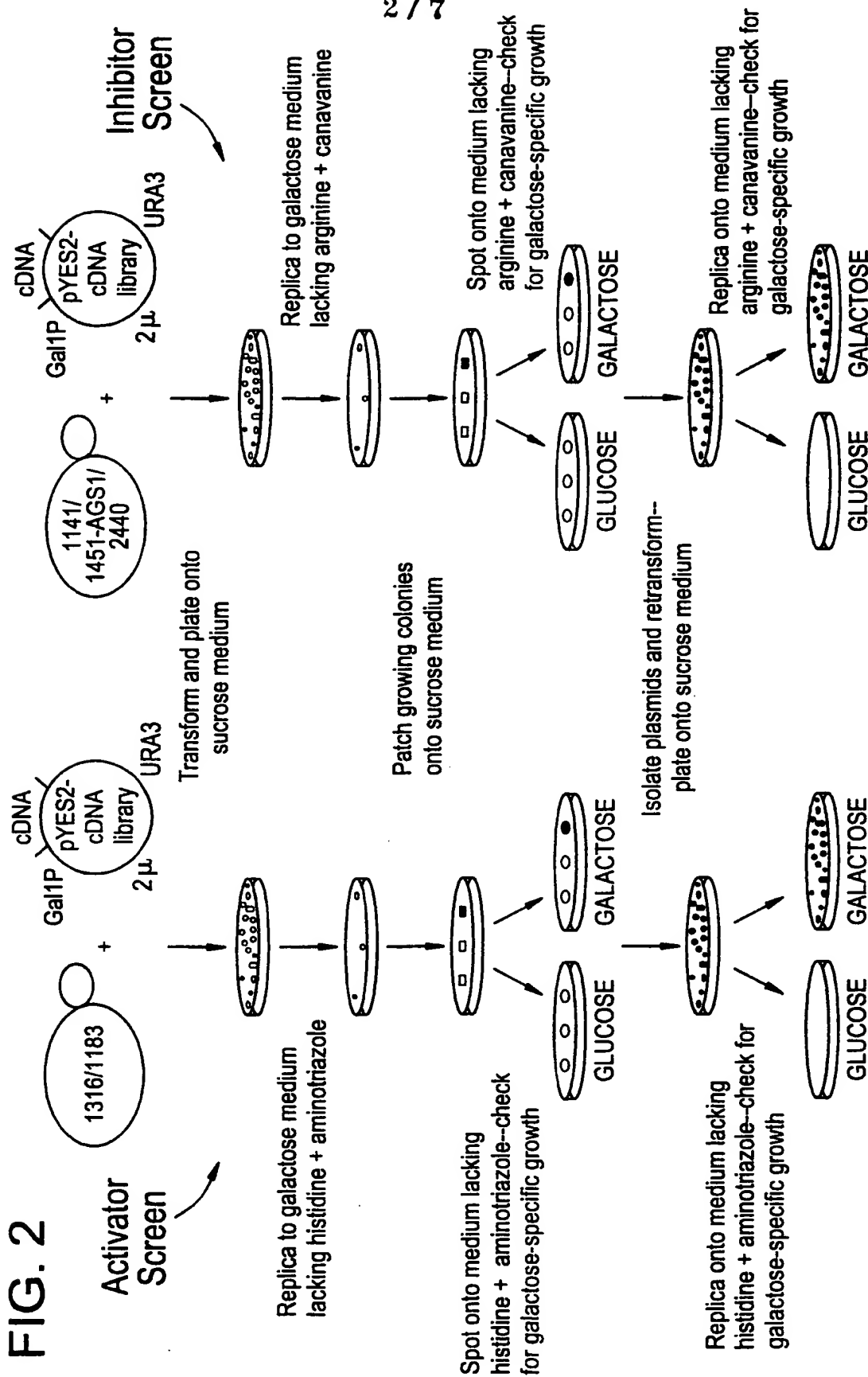


FIG. 3A

10	ATGA	ACTGG	CCGC	GATGAT	CAAGA	GATG	TGCC	CGAGCG	ACTCG	GAGCT	GAGTAT	CCCG	60
	GCCA	AGAACT	GCTAT	CGCAT	GGTCA	TCTTC	GGCT	CGTCCA	AGGTG	GGCAA	GACGG	CCATC	120
	GTGT	CGCGCT	TCCTC	ACCGG	CCGCT	TTCGAG	GACGC	CTACA	CGCCT	TACCAT	CGAGG	ACTTC	180
	CACCG	CAAGT	TCTACT	TCCAT	CCGCG	GCGAG	GTCTA	CCAGC	TCGAC	ATCCT	CGACA	CGTCC	240
	GGCA	ACCACC	CGTTCC	CCCGC	CATGC	GCGCG	CTCTC	CCATCC	TCACAG	GAGA	CGTTT	TCATC	300
310	CTGT	GTTC	A										360
	ATCC	TCGACA	CCAAG	TCTTG	CCTCA	AGAAC	AAACCA	CAAGG	AGAAC	CGTGGA	CGTGCC	CCCTG	420
	GTCA	TCTGG	GCAACA	AGGG	TGACC	GCGAC	TTCTA	CCCGG	AGGTG	GACCA	GCGCG	AGATC	480
	GAGC	AGCTGG	TGGGC	GACGA	CCCCA	GCGC	TGCGC	CTACT	TCGAG	ATCTC	GGCCA	AGAAG	540
	AACAG	CAGCC	TGGAC	CCAGAT	GTTC	CGCGC	CTCTT	CGCCA	TGGCC	AAAGCT	GCCC	AGCGAG	600
610	ATGAG	CCCCAG	ACCTG	CACCG	CAAGG	TCTCG	GTGC	AGTACT	GCGAC	GTGCT	GCACA	AGAAG	660
	GCGCT	GCGGA	ACAAG	AGCT	GCTGC	GGGCC	GGCAG	CGGCG	GCGCG	GGCGG	CGACC	CGGGC	720
	GACGC	CTTIG	GCATC	GTGGC	ACCTT	TCGCG	CGCCG	GCCCCA	GCGTA	CACAG	CGACCT	CATG	780
	TACAT	CCCGC	AGAAG	GCCAG	CGCCG	GCGAG	CAGGC	CAAGG	ACAAG	GAGCG	CTGCG	TCATC	840
	AGCTAG												

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FIG. 3A

10	20	30	40	50	60
ATGAAACTGG	CCGCGATGAT	CAAGAAGATG	TGCCCAGAGG	ACTCGGAGCT	GAGTATCCCG
60					
GCCAAAGAACT	GCTATCGCAT	GGTCATCCTC	GGCTCGTCCA	AGGTGGSCAA	GACGGCCATC
120					
GTGTCGCGCT	TCCTCACCGG	CCGCTTCGAG	GACGCCCTACA	CGCCTACCAT	CGAGGACTTC
180					
CACCGCAAGT	TCTACTCCAT	CCGCGGCGAG	GTCTACCAGC	TCGACATCCT	CGACACGTCC
240					
GGCAACCACC	CGTTCCCCGC	CATGCGGCGC	CTCTCCATCC	TCACAGGAGA	CGTTTTCATC
300					
310	320	330	340	350	360
CTGGTGTCA	GTCTGGACAA	CCGCGACTCC	TTCGAGGAGG	TGCAGCGGCT	CAGGCAGCAG
360					
ATCCTCGACA	CCAAGTCTTG	CCTCAAGAAC	AAAACCAAGG	AGAACGTGGA	CGTGCCCCCTG
420					
GTCACTCTCG	GCAACAAGGG	TGACCCGCGAC	TTCTACCCGG	AGGTGGACCA	GCGCGAGATC
480					
GAGCAGCTGG	TGGGCGACGA	CCCCCAGCGC	TGCGCCTACT	TCGAGATCTC	GGCCAAGAAG
540					
AACAGCAGCC	TGGACCAGAT	GTTCGCGCGG	CTCTTCGCCA	TGGCCAAGCT	GCCCAGCGAG
600					
610	620	630	640	650	660
ATGAGCCCCAG	ACCTGCACCG	CAAGGTCTCG	GTGCAGTACT	GCGACGTGCT	GCACAAGAAG
660					
GCGCTGCGGA	ACAAGAAGCT	GCTGCGGGCC	GGCAGCGGCG	GCGGCGGCGG	CGACCCGGGC
720					
GACGCCTTGG	GCATCGTGCG	ACCCCTCGCG	CGCCGGGCCA	GCGTACACAG	CGACCTCATG
780					
TACATCCGCG	AGAAGGCCAG	CGCCGGCAGC	CAGGCCAAGG	ACAAGGAGCG	CTGCGTTCATC
840					
AGCTAG	846				

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FIG.3B

MKLAAMIKKMCPSDSELSIP	AKNCYRMVILGSSKVGKTAI	40
	*	
VSRFLTGRFEDAYPTIEDF	HRKFYSIRGEVYQLDILDT	80
	*	
GNHPPAMRRLSILTGDVFI	LVFSLDNRDSFEVQRLRQQ	120
*		
ILDTKSLKNKTKENVIVPL	VICGNKGDREDFYREVDQREI	160
EQLVGDDPQRCAYFEISAKK	NSSLDQMFRALFAMAKLPSE	200
MSPDLHRKVSQYCDVLHKK	ALRNKKLLRAGSGGGGGDPG	240
DAFGIVAFARRPSVHSDLM	YIREKASAGSQAKDKERCVI	S 281

FIG.4A

	PM1	G1	PM2
C-HA-RAS1	-----MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDS		
RALA	-----MAANKPKGQNSLAHLKVTIMVSGGVGKSALTQLQFMDEYFDEYPTKADS		
RAB-1A	-----MSSMNPEYDYLFLKLLIGDSGVGKCLLLRFADDITYTESYISTIGVD		
RHOHP1	-----MTAAQAAGEEAPPGVRSVKVVLVGDGGCGKTSLLMVFADGAFPESTPTVFER		
CDC42	-----MQTIKCVVVGDAVGKTCLLISYTTNKFPPSEYVPTVFDN		
RAC2	-----MQAIKCVVVGDAVGKTCLLISYTTNKFPPSEYVPTVFDN		
ARL1	-----MGFFSSIFSLFGTREMRLILGLDGAGKTTILYRLQVGEVVTI-PTIGFN		
RND3/RHOE	-----MDPNQNVKCKIVVVGDSQCGKTALLHVFADKDCFPENYVPTVFEN		
AGS1	MKLAAMIKKMCPSDSELSIPAKNCYRMVILGSSKVGTAIVSRLTGRFEDAYTPTIEDF		
	: : : : *	: : : : *	: : : : *

	PM3
C-HA-RAS1	YRKQ-VVIDGETCLLDILDTAGQEEYSAMRDQYMRITGEGFLCVFAINNTKSFEDIHQYRE
RALA	YRKK-VVLDGEEVQIDILDTAGQEDYAAIRDNYFRSGEGFLCVFSITEMESFAATADFRE
RAB-1A	FKIRTIELDGKTIKLIQIWDTAGQERFRTITSSYYRGAHGIIVVDYDQESFNNVKQ-WL
RHOHP1	YMVN-LQVKGKPVHLHWDTAGQDDYDRLRPLFPDASVLLLCFDVTSFNSFDNIFNRWY
CDC42	YAVT-VMIGGEPTLGLFTAGQEDYDRLRPLSPQTDVFLVCFVSVPSSFFENVKEKWV
RAC2	YSAN-VMVDSKPVNLGLWDTAGQEDYDRLRPLSPQTDVFLICFSLVSPASYENVRAKWF
ARL1	VET----VTYKNLKFQVWDLGGQTSIRPYWRCYYSNDAVIYVVDSCDRDRIGISKSELV
RND3/RHOE	YTAS-FEIDTQRIELSLWDTSGSPYDNRVPLSYPDSDAVLICFDISRPTILDVLLKKWK
AGS1	HRKF-YSIRGEVYQLDILDTSGNHPPFAMRRRLSILTGDFILVFSLDNRDSFEVQRLRQ
	: : : : * . *

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FIG. 4B

G2

C-HA-RAS1
RALA
RAB-1A
RHOHP1
CDC42
RAC2
ARL1
RND3/RHOE
AGS1

QIKRVKD-----SDDVPMVLVGKCDLAA-----RTVESRQADLARS
QILRVKE-----DENVPFLLVGKNSDLEDK-----RQVSVEAKNRAEQ
QEIDRYA-----SENVKLLVGKNSDLETK-----KVVDYTTAKEFADS
PEVNH-----CKKVPILVVGCKTDLRKDKSLVKNKLRNGLEPVYHARGQEMARSV
PEITH-----CPKTPFLLVGTDIDLRDDPSTIEKLAKNKQKPIPTAEKLARDL
PEVRH-----CPSTPIILVGTKDLDRDDKDTIEKLKEKLAIPITYPQGLALAKEI
AMLEEE-----LRKAILVVFANKQDMEQAMTSSEMANSGLPALKORK-----
GEIOEF-----CPNTRMLLVGCKSDLRDVTSTLVLSNHRQTPVSYDQGANMAKQI
QILDTSCLKNKTENVDVPLVICGNKGD-RDFY-----REVDQREIEQLVGD

G3

C-HA-RAS1
RALA
RAB-1A
RHOHP1
CDC42
RAC2
ARL1
RND3/RHOE
AGS1

YG--IPYIETSAKTRQG-VEDAFYTLVREIR-----
WN--VNIVETSAKTRAN-VDRVFFDLREIR-----
LG--IPFLETSAKNATN-VEQSFMTMAAEIK-----
GA--VAYLECSARLHDN-VHAVFQEAEEVAL-----
KA--VKYVECSALTQRG-LKNVFDEAILAAL-----
DS--VKYLECSALTQRG-LKTVFDEAIRAVL-----
----WQIFKTSATKGTG-LDEAMEWLVTCLKSRQ
GA--ATYIECSALQSENSVRDIF-----HVATLAC-----VNKTNKNVKNK
DPQRCAYFEISAKKNSS-LDQMFRALEFAMAKLPSEMSPDLHRKVSQYCDVLHKKALRNKK

... : : * *

C-HA-RAS1
RALA
RAB-1A
RHOHP1
CDC42
RAC2
ARL1
RND3/RHOE
AGS1

-----QHKLRKLNPPDESGPGCMSCKCVLS
-----ARKMEDSKERNGKKRKSIAKIRERCCIL
-KRMGPGATAGGA-----EKS NVKIQTSTPVKQAGGGCC
-----SSRGRNFWRRITQGFVVT
-----EPPETQPKRKCCIF
-----CPQPTRQQKRACSLI

SQRA-----TKRISHMPSRP-----ELSAVATDLRKDKAKSCTVM
LLRAGSGGGGDPGDAFGIVAFARRPSVHSDLMYIREKASAGSQAOKKERCVIS

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FIG.5

	P REGION			G' REGION	
RhoE/Rnd3	KIVVVGDSQCGKTALL	.	.	.	LSLWDTSGSPYYD
Rnd2	KIVVVGDAECGKTALL	.	.	.	LNWMDTSGSSYYD
Rnd1	KLVLVGDVQCGKTAML	.	.	.	LSLWDTSGSPYYD
RhoA	KLIVVGDGACGKTCLL	.	.	.	LALWDTAGQEDYD
RhoB	KLWVVGDGACGKTCLL	.	.	.	LALWDTAGQEDYD
Cdc42	KCVVVGDGAVGKTCLL	.	.	.	LGLFDTAGQEDYD
Rac1	KCVVVGDGAVGKTCLL	.	.	.	LGLWDTAGQEDYD
H-ras	KLWVVGAGGVGKSALT	.	.	.	LDILDTAGQEEYD
AGS	26 RMVILGSSSKVGKTAIV	.	.	.	LDILDTSGNHPPF 86
					* *

- 1 -

SEQUENCE LISTING

<110> Duzic, Emir et al.

5 <120> AGS PROTEINS AND NUCLEIC ACID MOLECULES AND USES THEREOF

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<170> PatentIn Ver. 2.0

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1				5					10					15		

ctg	agt	atc	ccg	gcc	aag	aac	tgc	tat	cgc	atg	gtc	atc	ctc	ggc	tcg	96
Leu	Ser	Ile	Pro	Ala	Lys	Asn	Cys	Tyr	Arg	Met	Val	Ile	Leu	Gly	Ser	
			20					25					30			

tcc	aag	gtg	ggc	aag	acg	gcc	atc	gtg	tcg	cgc	ttc	ctc	acc	ggc	cgc	144
Ser	Lys	Val	Gly	Lys	Thr	Ala	Ile	Val	Ser	Arg	Phe	Leu	Thr	Gly	Arg	
		35					40					45				

ttc	gag	gac	gcc	tac	acg	cct	acc	atc	gag	gac	ttc	cac	cgc	aag	ttc	192
Phe	Glu	Asp	Ala	Tyr	Thr	Pro	Thr	Ile	Glu	Asp	Phe	His	Arg	Lys	Phe	
40		50				55					60					

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Tyr	Ser	Ile	Arg	Gly	Glu	Val	Tyr	Gln	Leu	Asp	Ile	Leu	Asp	Thr	Ser	
		65			70				75					80		

ggc	aac	cac	ccg	ttc	ccc	gcc	atg	cgg	cgc	ctc	tcc	atc	ctc	aca	gga	288
Gly	Asn	His	Pro	Phe	Pro	Ala	Met	Arg	Arg	Leu	Ser	Ile	Leu	Thr	Gly	
			85					90						95		

gac	gtt	ttc	atc	ctg	gtg	ttc	agt	ctg	gac	aac	cgc	gac	tcc	ttc	gag	336
Asp	Val	Phe	Ile	Leu	Val	Phe	Ser	Leu	Asp	Asn	Arg	Asp	Ser	Phe	Glu	
			100					105					110			

gag	gtg	cag	cgg	ctc	agg	cag	cag	atc	ctc	gac	acc	aag	tct	tgc	ctc	384
Glu	Val	Gln	Arg	Leu	Arg	Gln	Gln	Ile	Leu	Asp	Thr	Lys	Ser	Cys	Leu	
		115				120						125				

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Lys	Asn	Lys	Thr	Lys	Glu	Asn	Val	Asp	Val	Pro	Leu	Val	Ile	Cys	Gly	
60		130				135					140					

aac	aag	ggt	gac	cgc	gac	ttc	tac	cgc	gag	gtg	gac	cag	cgc	gag	atc	480
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

- 2 -

Asn Lys Gly Asp Arg Asp Phe Tyr Arg Glu Val Asp Gln Arg Glu Ile
 145 150 155 160

gag cag ctg gtg ggc gac gac ccc cag cgc tgc gcc tac ttc gag atc 528
 5 Glu Gln Leu Val Gly Asp Asp Pro Gln Arg Cys Ala Tyr Phe Glu Ile
 165 170 175

tcg gcc aag aag aac agc agc ctg gac cag atg ttc cgc gcg ctc ttc 576
 10 Ser Ala Lys Lys Asn Ser Ser Leu Asp Gln Met Phe Arg Ala Leu Phe
 180 185 190

gcc atg gcc aag ctg ccc agc gag atg agc cca gac ctg cac cgc aag 624
 Ala Met Ala Lys Leu Pro Ser Glu Met Ser Pro Asp Leu His Arg Lys
 195 200 205

15 gtc tcg gtg cag tac tgc gac gtg ctg cac aag aag gcg ctg cgg aac 672
 Val Ser Val Gln Tyr Cys Asp Val Leu His Lys Lys Ala Leu Arg Asn
 210 215 220

20 aag aag ctg ctg cgg gcc ggc agc ggc ggc ggc ggc gac ccg ggc 720
 Lys Lys Leu Leu Arg Ala Gly Ser Gly Gly Gly Gly Gly Asp Pro Gly
 225 230 235 240

gac gcc ttt ggc atc gtg gca ccc ttc gcg cgc cgg ccc agc gta cac 768
 25 Asp Ala Phe Gly Ile Val Ala Pro Phe Ala Arg Arg Pro Ser Val His
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agc gac ctc atg tac atc cgc gag aag gcc agc gcc ggc agc cag gcc 816
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 20 25 30

Ser Lys Val Gly Lys Thr Ala Ile Val Ser Arg Phe Leu Thr Gly Arg
 50 35 40 45

Phe Glu Asp Ala Tyr Thr Pro Thr Ile Glu Asp Phe His Arg Lys Phe
 50 55 60

55 Tyr Ser Ile Arg Gly Glu Val Tyr Gln Leu Asp Ile Leu Asp Thr Ser
 65 70 75 80

Gly Asn His Pro Phe Pro Ala Met Arg Arg Leu Ser Ile Leu Thr Gly
 85 90 95

60 Asp Val Phe Ile Leu Val Phe Ser Leu Asp Asn Arg Asp Ser Phe Glu
 100 105 110

- 3 -

Glu Val Gln Arg Leu Arg Gln Gln Ile Leu Asp Thr Lys Ser Cys Leu
 115 120 125
 5 Lys Asn Lys Thr Lys Glu Asn Val Asp Val Pro Leu Val Ile Cys Gly
 130 135 140
 Asn Lys Gly Asp Arg Asp Phe Tyr Arg Glu Val Asp Gln Arg Glu Ile
 145 150 155 160
 10 Glu Gln Leu Val Gly Asp Asp Pro Gln Arg Cys Ala Tyr Phe Glu Ile
 165 170 175
 Ser Ala Lys Lys Asn Ser Ser Leu Asp Gln Met Phe Arg Ala Leu Phe
 15 180 185 190
 Ala Met Ala Lys Leu Pro Ser Glu Met Ser Pro Asp Leu His Arg Lys
 195 200 205
 20 Val Ser Val Gln Tyr Cys Asp Val Leu His Lys Lys Ala Leu Arg Asn
 210 215 220
 Lys Lys Leu Leu Arg Ala Gly Ser Gly Gly Gly Gly Gly Asp Pro Gly
 225 230 235 240
 25 Asp Ala Phe Gly Ile Val Ala Pro Phe Ala Arg Arg Pro Ser Val His
 245 250 255
 Ser Asp Leu Met Tyr Ile Arg Glu Lys Ala Ser Ala Gly Ser Gln Ala
 30 260 265 270
 Lys Asp Lys Glu Arg Cys Val Ile Ser
 275 280
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 ccagccgtc accccggtg ccacccagc gaccctcagc cgtctctgc cttctctcg 120
 50 gccccgcgc cgcctcgcg gccctctgc cca atg aaa ctg gcc gcg atg atc 174
 Met Lys Leu Ala Ala Met Ile
 1 5
 aag aag atg tgc ccg agc gac tcg gag ctg agt atc ccg gcc aag aac 222
 55 Lys Lys Met Cys Pro Ser Asp Ser Glu Leu Ser Ile Pro Ala Lys Asn
 10 15 20
 tgc tat cgc atg gtc atc ctc ggc tcg tcc aag gtg ggc aag acg gcc 270
 60 Cys Tyr Arg Met Val Ile Leu Gly Ser Ser Lys Val Gly Lys Thr Ala
 25 30 35
 atc gtg tcg cgc ttc ctc acc ggc cgc ttc gag gac gcc tac acg cct 318

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	Ile Val Ser Arg Phe Leu Thr Gly Arg Phe Glu Asp Ala Tyr Thr Pro	
	40 45 50 55	
5	acc atc gag gac ttc cac cgc aag ttc tac tcc atc cgc ggc gag gtc Thr Ile Glu Asp Phe His Arg Lys Phe Tyr Ser Ile Arg Gly Glu Val	366
	60 65 70	
10	tac cag ctc gac atc ctc gac acg tcc ggc aac cac ccg ttc ccc gcc Tyr Gln Leu Asp Ile Leu Asp Thr Ser Gly Asn His Pro Phe Pro Ala	414
	75 80 85	
15	atg cgg cgc ctc tcc atc ctc aca gga gac gtt ttc atc ctg gtg ttc Met Arg Arg Leu Ser Ile Leu Thr Gly Asp Val Phe Ile Leu Val Phe	462
	90 95 100	
20	agt ctg gac aac cgc gac tcc ttc gag gag gtg cag cgg ctc agg cag Ser Leu Asp Asn Arg Asp Ser Phe Glu Glu Val Gln Arg Leu Arg Gln	510
	105 110 115	
25	cag atc ctc gac acc aag tct tgc ctc aag aac aaa acc aag gag aac Gln Ile Leu Asp Thr Lys Ser Cys Leu Lys Asn Lys Thr Lys Glu Asn	558
	120 125 130 135	
30	gtg gac gtg ccc ctg gtc atc tgc ggc aac aag ggt gac cgc gac ttc Val Asp Val Pro Leu Val Ile Cys Gly Asn Lys Gly Asp Arg Asp Phe	606
	140 145 150	
35	tac cgc gag gtg gac cag cgc gag atc gag cag ctg gtg ggc gac gac Tyr Arg Glu Val Asp Gln Arg Glu Ile Glu Gln Leu Val Gly Asp Asp	654
	155 160 165	
40	ccc cag cgc tgc gcc tac ttc gag atc tgc gcc aag aag aac agc agc Pro Gln Arg Cys Ala Tyr Phe Glu Ile Ser Ala Lys Lys Asn Ser Ser	702
	170 175 180	
45	ctg gac cag atg ttc cgc gcg ctc ttc gcc atg gcc aag ctg ccc agc Leu Asp Gln Met Phe Arg Ala Leu Phe Ala Met Ala Lys Leu Pro Ser	750
	185 190 195	
50	gag atg agc cca gac ctg cac cgc aag gtc tgc gtg cag tac tgc gac Glu Met Ser Pro Asp Leu His Arg Lys Val Ser Val Gln Tyr Cys Asp	798
	200 205 210 215	
55	gtg ctg cac aag aag gcg ctg cgg aac aag aag ctg ctg cgg gcc ggc Val Leu His Lys Lys Ala Leu Arg Asn Lys Lys Leu Leu Arg Ala Gly	846
	220 225 230	
60	agc ggc ggc ggc ggc ggc gac ccg ggc gac gcc ttt ggc atc gtg gca Ser Gly Gly Gly Gly Asp Pro Gly Asp Ala Phe Gly Ile Val Ala	894
	235 240 245	
65	ccc ttc gcg cgc cgg ccc agc gta cac agc gac ctc atg tac atc cgc Pro Phe Ala Arg Arg Pro Ser Val His Ser Asp Leu Met Tyr Ile Arg	942
	250 255 260	
70	gag aag gcc agc gcc ggc agc cag gcc aag gac aag gag cgc tgc gtc Glu Lys Ala Ser Ala Gly Ser Gln Ala Lys Asp Lys Glu Arg Cys Val	990
	265 270 275	
75	atc agc taggagcccc gccgcgctgg cgacacaacc taaggaggac ctttttgtta Ile Ser	1046
	280	

- 5 -

agtc aaatcc aacggcccgg tgcgccccag gccgggagcg cgcgcggact ggcgtctccc 1106
ctccccggcg tccgccccca gcactgggga ggcgccactg aaccgagaag ggacgggtcat 1166
5 ctgctccgga aggaaagaga acgggccaag actgggacta tccccaccc ccggtccccc 1226
attgaggccc gccaccccc aacttttggg agcgaggggc cagccgaggg tggatttatc 1286
10 ttctcaaaga cctaagagtg agcgcggggt gggggaggga tgtgaagtta tccagcctct 1346
gctaggcttc aagaaaccgt catgcccgt tgagggtcag gacccacggg gcattatctt 1406
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15 ggggggctg ggtcaaatca tagccaagtg acttgtttac atgtgagtga aactgcacaa 1526
aggaacacaa aacaaaactt gcactttaac ggtagtccg gtgtcaacat ggacacgaac 1586
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ttggtttttt aatatacaat aaaataattt aaatggaaa aaaaaaaaaa aaaaaaaaaa 1706
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aagcgccgc tcgagcatgc 1766
25 atctagaggg ccgcatcatg taattagtta tgaac 1801

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35 <223> Probe/Primer

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ctcatggagc tcaaaactgtt actattaggt gccg 34

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<211> 54
<212> DNA
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45 <223> Probe/Primer

<400> 5
ctgctggtcg acgcggccgc tcatataata ccaatttttt taagggtttg ctgg 54

50
<210> 6
<211> 28
<212> DNA
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55 <223> Probe/Primer

<400> 6
gtttgatgtg ggtgctcagc ggtctgag 28

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<210> 7
<211> 29

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<212> DNA
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ctcagaaccg ctgagcaccc acatcaaac 29

<210> 8
10 <211> 35
<212> DNA
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15 <400> 8
cgggatccat gaaactggcc gcgatgatca agaag 35

<210> 9
20 <211> 28
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25 <400> 9
ggaattccta gctgatgacg cagcgctc 28

<210> 10
30 <211> 25
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35 <400> 10
ggatccatgc aaacgctaaa gtgtg 25

<210> 11
40 <211> 31
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45 <400> 11
gaattcgact acaaaattgc acatttttta c 31

<210> 12
50 <211> 31
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<213> Artificial sequence
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55 <400> 12
cgcatggtca tcctcgtttc gtccaagggtg g 31

<210> 13
60 <211> 31
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<223> Probe/Primer

<400> 13
 ccaccttgga cgaaacgagg atgaccatgc g 31
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<210> 14
 <211> 35
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 10 <213> Artificial sequence
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<400> 14
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<210> 16
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 30 <213> Artificial sequence
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 35

<210> 17
 <211> 32
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 40 <213> Artificial sequence
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 cggggaacgg gtggtagcg gacgtgtcga gg 32
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<210> 18
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 50 <213> Unknown

<220> Xaa's at positions 2-5 may be any amino acid; Xaa at position 8 may be Serine or Threonine acid

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 Gly Xaa Xaa Xaa Xaa Gly Lys Xaa
 1 5

60
 <210> 19
 <211> 4

- 8 -

<212> PRT
<213> Unknown

<220> Xaa's at positions 2,3 may be any amino acid

5 <400> 19
Asp Xaa Xaa Gly
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10 <210> 20
<211> 4
<212> PRT
<213> Unknown

15 <220> Xaa at position 3 may be any amino acid

<400> 20
Asn Lys Xaa Asp
20 1

<210> 21
<211> 5

25 <212> PRT
<213> Unknown

<220> Xaa at position 2 may be any amino acid

30 <400> 21
Glu Xaa Ser Ala Lys
1 5

35 <210> 22
<211> 4
<212> PRT
<213> Unknown

40 <220> Xaa at position 2 or 3 may be any aliphatic amino acid residue
<220> Xaa at position 4 may be any amino acid

<400> 22
Cys Xaa Xaa Xaa
45 1

<210> 23
<211> 7

50 <212> PRT
<213> Homo sapiens

<400> 23
Gln Ala Lys Asp Lys Glu Arg
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<210> 24
<211> 1691

60 <212> DNA
<213> Homo sapiens

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<220>

<221> CDS

<222> (45) .. (587)

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Met Cys Lys Gly
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10 ctt gca gct ttg ccc cac tca tgc ctg gaa agg gcc aag gag att aag 104
Leu Ala Ala Leu Pro His Ser Cys Leu Glu Arg Ala Lys Glu Ile Lys
5 10 15 20

atc aag ttg gga att ctc ctc cag aag cca gac tca gtt ggt gac ctt 152
15 Ile Lys Leu Gly Ile Leu Leu Gln Lys Pro Asp Ser Val Gly Asp Leu
25 30 35

gtc att ccg tac aat gag aag cca gag aaa cca gcc aag acc cag aaa 200
20 Val Ile Pro Tyr Asn Glu Lys Pro Glu Lys Pro Ala Lys Thr Gln Lys
40 45 50

acc tcg ctg gac gag gcc ctg cag tgg cgt gat tcc ctg gac aaa ctc 248
Thr Ser Leu Asp Glu Ala Leu Gln Trp Arg Asp Ser Leu Asp Lys Leu
55 60 65

25 ctg cag aac aac tat gga ctt gcc agt ttc aaa agt ttc ctg aag tct 296
Leu Gln Asn Asn Tyr Gly Leu Ala Ser Phe Lys Ser Phe Leu Lys Ser
70 75 80

30 gaa ttc agt gag gaa aac ctt gag ttc tgg att gcc tgt gag gat tac 344
Glu Phe Ser Glu Glu Asn Leu Glu Phe Trp Ile Ala Cys Glu Asp Tyr
85 90 95 100

aag aag atc aag tcc cct gcc aag atg gct gag aag gca aag caa att 392
35 Lys Lys Ile Lys Ser Pro Ala Lys Met Ala Glu Lys Ala Lys Gln Ile
105 110 115

tat gaa gaa ttc att caa acg gag gct cct aaa gag gtg aat att gac 440
40 Tyr Glu Glu Phe Ile Gln Thr Glu Ala Pro Lys Glu Val Asn Ile Asp
120 125 130

cac ttc act aag gac atc aca atg aag aac ctg gtg gaa cct tcc ctg 488
His Phe Thr Lys Asp Ile Thr Met Lys Asn Leu Val Glu Pro Ser Leu
135 140 145

45 agc agc ttt gac atg gcc cag aaa aga atc cat gcc ctg atg gaa aag 536
Ser Ser Phe Asp Met Ala Gln Lys Arg Ile His Ala Leu Met Glu Lys
150 155 160

50 gat tct ctg cct cgc ttt gtg cgc tct gag ttt tat cag gag tta atc 584
Asp Ser Leu Pro Arg Phe Val Arg Ser Glu Phe Tyr Gln Glu Leu Ile
165 170 175 180

aag tagtaattta gccaggctat gaaatcatcc tgtgagttat ttctccata 637
55 Lys

ataaccctgc atttccatt aatctacata tcttccaca gcagctttgc tcagtgtatc 697

ccacatggga aaaatccag gggatgttgc ttactctttt tgcccacact gctttggata 757
60 cttatctact gtccgaagge cttctttccc cactcaattc ttctgccct gttattaatt 817

- 10 -

aagatatctt cagcttgtag tcagacccaa tcagaatcac agaaaaatcc tgcctaaggc 877
 aaagaaatat aagacaagac tatgatatca atgaatgtgg gttaagtaat agatttccag 937
 5 ctaaattggt ctaaaaaaga atattaagtg tggacagacc tatttcaaag gagcttaatt 997
 gatctcactt gttttagttc tgatccaggg agatcacccc tctaattatt tctgaacttg 1057
 gttaataaaa gtttataaga tttttatgaa gcagccactg tatgatattt taagcaaata 1117
 10 tgttatttaa aatattgatc cttcccttgg accaccttca tgttagttgg gtattataaa 1177
 taagagatac aacctgaat atattatggt tatacaaat caatctgaac acaattcata 1237
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 ccacctaata atcaagatgg ctatatgcta aaaagagaaa atatggtcaa gtctaaaatg 1537
 25 gctaattgtc ctatgatgct attatcatag actaatgaca tttatcttca aaacaccaa 1597
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<211> 181

<212> PRT

35 <213> Homo sapiens

<400> 25

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 40 Lys Glu Ile Lys Ile Lys Leu Gly Ile Leu Leu Gln Lys Pro Asp Ser
 20 25 30
 Val Gly Asp Leu Val Ile Pro Tyr Asn Glu Lys Pro Glu Lys Pro Ala
 45 35 40 45
 Lys Thr Gln Lys Thr Ser Leu Asp Glu Ala Leu Gln Trp Arg Asp Ser
 50 55 60
 50 Leu Asp Lys Leu Leu Gln Asn Asn Tyr Gly Leu Ala Ser Phe Lys Ser
 65 70 75 80
 Phe Leu Lys Ser Glu Phe Ser Glu Glu Asn Leu Glu Phe Trp Ile Ala
 85 90 95
 55 Cys Glu Asp Tyr Lys Lys Ile Lys Ser Pro Ala Lys Met Ala Glu Lys
 100 105 110
 60 Ala Lys Gln Ile Tyr Glu Glu Phe Ile Gln Thr Glu Ala Pro Lys Glu
 115 120 125
 Val Asn Ile Asp His Phe Thr Lys Asp Ile Thr Met Lys Asn Leu Val

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	130	135	140	
	Glu Pro Ser Leu Ser Ser Phe Asp Met Ala Gln Lys Arg Ile His Ala			
	145	150	155	160
5	Leu Met Glu Lys Asp Ser Leu Pro Arg Phe Val Arg Ser Glu Phe Tyr			
		165	170	175
	Gln Glu Leu Ile Lys			
10		180		
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	<210> 27			
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	<210> 29			
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	<400> 29			
50	ccgctcgagt taggcacact gagggacc			28
	<210> 30			
	<211> 41			
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	<213> Homo sapiens			
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60	agtcggtacc cgcatagatc tgcaggatgc cctttttgac g			41
	<210> 31			

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<211> 34
<212> DNA
<213> Artificial sequence
<223> Probe/Primer

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10 <210> 32
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<400> 32
tggcctcgag atgacaaatt caaaagaaga cg 32

20 <210> 33
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25
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atcactgcag ctatgctaca acattccaaa at 32

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gggtcatgaa actggccgcg atgatcaaga ag 32

40 <210> 35
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gatagtcgac ctagctgatg acgcagcgct c 31

50 <210> 36
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60 <210> 37
<211> 31
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<213> Artificial sequence
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 <210> 38
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 25 ctagctgatg acgctgcgct ccttgctcctt gg 32

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 30 <212> DNA
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 35 <222> (1)..(834)

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 ttg tcc agc ggg aac tgc acg ctc agt gtg ccc gcc aaa aac tca tac 96
 Leu Ser Ser Gly Asn Cys Thr Leu Ser Val Pro Ala Lys Asn Ser Tyr
 20 25 30
 45
 cgc atg gtg gtg ctg ggt gcc tct cgg gtg ggc aag agc tcc atc gtg 144
 Arg Met Val Val Leu Gly Ala Ser Arg Val Gly Lys Ser Ser Ile Val
 35 40 45
 50 tct cgc ttc ctc aat ggc cgc ttt gag gac cag tac aca ccc acc atc 192
 Ser Arg Phe Leu Asn Gly Arg Phe Glu Asp Gln Tyr Thr Pro Thr Ile
 50 55 60
 gag gac ttc cac cgt aag gta tac aac atc cgc gcc gac atg tac cag 240
 Glu Asp Phe His Arg Lys Val Tyr Asn Ile Arg Gly Asp Met Tyr Gln
 65 70 75 80
 60 ctc gac atc ctg gat acc tct ggc aac cac ccc ttc ccc gcc atg cgc 288
 Leu Asp Ile Leu Asp Thr Ser Gly Asn His Pro Phe Pro Ala Met Arg
 85 90 95
 agg ctg tcc atc ctc aca ggg gat gtc ttc atc ctg gtg ttc agc ctg 336

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	Arg	Leu	Ser	Ile	Leu	Thr	Gly	Asp	Val	Phe	Ile	Leu	Val	Phe	Ser	Leu	
				100					105					110			
	gat	aac	cgg	gag	tcc	ttc	gat	gag	gtc	aag	cgc	ctt	cag	aag	cag	atc	384
5	Asp	Asn	Arg	Glu	Ser	Phe	Asp	Glu	Val	Lys	Arg	Leu	Gln	Lys	Gln	Ile	
			115					120					125				
	ctg	gag	gtc	aag	tcc	tgc	ctg	aag	aac	aag	acc	aag	gag	gcg	gcg	gag	432
10	Leu	Glu	Val	Lys	Ser	Cys	Leu	Lys	Asn	Lys	Thr	Lys	Glu	Ala	Ala	Glu	
		130					135					140					
	ctg	ccc	atg	gtc	atc	tgt	ggc	aac	aag	aac	gac	cac	ggc	gag	ctg	tgc	480
15	Leu	Pro	Met	Val	Ile	Cys	Gly	Asn	Lys	Asn	Asp	His	Gly	Glu	Leu	Cys	
	145					150				155					160		
	cgc	cag	gtg	ccc	acc	acc	gag	gcc	gag	ctg	ctg	gtg	tcg	ggc	gac	gag	528
	Arg	Gln	Val	Pro	Thr	Thr	Glu	Ala	Glu	Leu	Leu	Val	Ser	Gly	Asp	Glu	
				165						170					175		
20	aac	tgc	gcc	tac	ttc	gag	gtg	tcg	gcc	aag	aag	aac	acc	aac	gtg	gac	576
	Asn	Cys	Ala	Tyr	Phe	Glu	Val	Ser	Ala	Lys	Lys	Asn	Thr	Asn	Val	Asp	
				180					185					190			
	gag	atg	ttc	tac	gtg	ctc	ttc	agc	atg	gcc	aag	ctg	cca	cac	gag	atg	624
25	Glu	Met	Phe	Tyr	Val	Leu	Phe	Ser	Met	Ala	Lys	Leu	Pro	His	Glu	Met	
		195						200					205				
	agc	ccc	gcc	ctg	cat	cgc	aag	atc	tcc	gtg	cag	tac	ggg	gac	gcc	ttc	672
30	Ser	Pro	Ala	Leu	His	Arg	Lys	Ile	Ser	Val	Gln	Tyr	Gly	Asp	Ala	Phe	
		210					215					220					
	cac	ccc	agg	ccc	ttc	tgc	atg	cgc	cgc	gtc	aag	gag	atg	gac	gcc	tat	720
35	His	Pro	Arg	Pro	Phe	Cys	Met	Arg	Arg	Val	Lys	Glu	Met	Asp	Ala	Tyr	
	225					230					235				240		
	ggc	atg	gtc	tcg	ccc	ttc	gcc	cgc	cgc	ccc	agc	gtc	aac	agt	gac	ctc	768
	Gly	Met	Val	Ser	Pro	Phe	Ala	Arg	Arg	Pro	Ser	Val	Asn	Ser	Asp	Leu	
					245					250					255		
40	aag	tac	atc	aag	gcc	aag	gtc	ctt	cgg	gaa	ggc	cag	gcc	cgt	gag	agg	816
	Lys	Tyr	Ile	Lys	Ala	Lys	Val	Leu	Arg	Glu	Gly	Gln	Ala	Arg	Glu	Arg	
				260					265					270			
	gac	aag	tgc	acc	atc	cag	tga										837
45	Asp	Lys	Cys	Thr	Ile	Gln											
			275														
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	1				5					10					15		
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				20					25					30			
60	Arg	Met	Val	Val	Leu	Gly	Ala	Ser	Arg	Val	Gly	Lys	Ser	Ser	Ile	Val	
			35					40					45				

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Ser Arg Phe Leu Asn Gly Arg Phe Glu Asp Gln Tyr Thr Pro Thr Ile
 50 55 60
 5 Glu Asp Phe His Arg Lys Val Tyr Asn Ile Arg Gly Asp Met Tyr Gln
 65 70 75 80
 Leu Asp Ile Leu Asp Thr Ser Gly Asn His Pro Phe Pro Ala Met Arg
 85 90 95
 10 Arg Leu Ser Ile Leu Thr Gly Asp Val Phe Ile Leu Val Phe Ser Leu
 100 105 110
 Asp Asn Arg Glu Ser Phe Asp Glu Val Lys Arg Leu Gln Lys Gln Ile
 115 120 125
 15 Leu Glu Val Lys Ser Cys Leu Lys Asn Lys Thr Lys Glu Ala Ala Glu
 130 135 140
 20 Leu Pro Met Val Ile Cys Gly Asn Lys Asn Asp His Gly Glu Leu Cys
 145 150 155 160
 Arg Gln Val Pro Thr Thr Glu Ala Glu Leu Leu Val Ser Gly Asp Glu
 165 170 175
 25 Asn Cys Ala Tyr Phe Glu Val Ser Ala Lys Lys Asn Thr Asn Val Asp
 180 185 190
 Glu Met Phe Tyr Val Leu Phe Ser Met Ala Lys Leu Pro His Glu Met
 195 200 205
 30 Ser Pro Ala Leu His Arg Lys Ile Ser Val Gln Tyr Gly Asp Ala Phe
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 35 His Pro Arg Pro Phe Cys Met Arg Arg Val Lys Glu Met Asp Ala Tyr
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Glu Met Glu Ser Phe Ala Ala Thr Ala Asp Phe Arg Glu Gln Ile Leu
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60 Thr Thr Lys Lys Val Val Asp Tyr Thr Thr Ala Lys Glu Phe Ala Asp
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 Lys Pro Val Asn Leu Gly Leu Trp Asp Thr Ala Gly Gln Glu Asp Tyr
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 Asp Arg Leu Arg Pro Leu Ser Tyr Pro Gln Thr Asp Val Phe Leu Ile

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	Thr Gln Arg Gly Leu Lys Thr Val Phe Asp Glu Ala Ile Arg Ala Val						
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Gly Ser Pro Tyr Tyr Asp Asn Val Arg Pro Leu Ser Tyr Pro Asp Ser
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 99/10151

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/12 C12N15/11 C12N15/62 C12Q1/02 C12Q1/68 C07K14/47 C07K16/18 A01K67/027 A61K38/17 A61K39/395 A61K48/00 G01N33/68 G01N33/50					
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12Q C07K G01N					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category *	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	MARRA ET AL.: "The WashU-HHMI Mouse EST project" EMBEST3:AA790463, 8 February 1998 (1998-02-08), XP002113557 the whole document				1,2,4,5,8-11
X	KEMPPAINEN ET AL.: "Dexamethasone rapidly induces a novel Ras superfamily member-related gene in AtT-20 cells" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 6, 6 February 1998 (1998-02-06), pages 3129-3131, XP002113558 page 3129 - page 3131; figures 1-3 --- -/--				1-18, 20-23, 25-29, 52,53
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.					
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family					
Date of the actual completion of the international search 30 August 1999			Date of mailing of the international search report 13/09/1999		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016			Authorized officer van Klompenburg, W		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/10151

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Y -S KANG ET AL: "Effects of expression of mammalian Galpha and hybrid mammalian yeast Galpha proteins on the yeast pheromones response signal transduction pathway" MOLECULAR AND CELLULAR BIOLOGY, vol. 10, no. 6, 1 June 1990 (1990-06-01), pages 2582-2590, XP002092715 ISSN: 0270-7306 page 742-746; figures 3,4; table 2	30, 34-36, 38, 42-46, 55-59,66
X	DRUEY ET AL: "INHIBITION OF G-PROTEIN-MEDIATED MAP KINASE ACTIVATION BY A NEW MAMMALIAN GENE FAMILY" NATURE, vol. 379, no. 6567, 1 January 1996 (1996-01-01), pages 742-746, XP002058369 ISSN: 0028-0836 the whole document	30,42, 43,47, 72-78
X	WO 94 13802 A (CYTOMED INC ;BETH ISRAEL HOSPITAL (US)) 23 June 1994 (1994-06-23) page 40-46; claims 1-23	19,30, 39,42-54
X	LI ET AL.: "Substitutions in the pheromone-responsive Gb protein of Saccharomyces cerevisiae confer a defect in recovery from pheromone treatment" GENETICS, vol. 148, no. 3, 1 March 1998 (1998-03-01), pages 947-961, XP002113559 page 950 - page 958; figures 1,4,5,7; table 1	55-59,66
X	CHEN ET AL.: "Characterization of a novel mammalian RGS protein that binds to Ga proteins and inhibits pheromone signalling in yeast" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 13, 28 March 1997 (1997-03-28), pages 8679-8685, XP002113560 the whole document	30,72
A		32,36, 39, 55-59, 66,73-78
X	WO 94 23025 A (CADUS PHARMACEUTICALS INC) 13 October 1994 (1994-10-13) page 12 - page 17; claims 33-36 page 42 - page 43	30,32,36
A		37
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/10151

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,P, X	CISMOWSKI ET AL.: "A yeast-based approach to functional cloning of novel mammalian proteins in the G-protein signaling pathway" NAUNYN-SCHMIEDEBERGS ARCHIVES OF PHARMACOLOGY, vol. 358, no. 1 suppl 2, July 1998 (1998-07), page p2137 XP002113561 the whole document	1-78
P,X	TAKESONO ET AL: "Stimulus input to heterotimeric G-protein signalling pathways" FASEB JOURNAL, vol. 13, no. 5, 15 March 1999 (1999-03-15), page a796 XP002113562 the whole document	1-78
T	WO 99 18211 A (BENEGAL ANUPAMA N ;CADUS PHARMACEUTICAL CORP (US); KLEIN CHRISTINE) 15 April 1999 (1999-04-15) claims 27-43,94-108; examples 4,11	1-78

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 10151

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 42-51
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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